IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE: :

U.S. PATENT NO. 5,270,057 :

ISSUED: December 14, 1993 :

TO: Andreas L.J. de Meere and Marinus A. De Ruiter : RECEIVED

FOR: STABILIZED GONADOTROPIN CONTAINING PREPARATIONS : NOV 2 5 1997

PATENT EXTENSION
FROM: SERIAL NO. 914,227 : A/C PATENTS

OF: July 3, 1992 :

TRANSMITTAL LETTER FOR APPLICATION FOR
EXTENSION OF PATENT TERM UNDER 35 U.S.C. §156

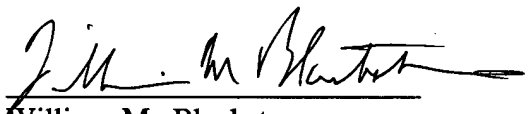
Assistant Commissioner of Patents and Trademarks
Box Patent Extension
Washington, D.C. 20231

Sir:

Transmitted herewith is the application of Akzo Nobel N.V. for extension of the term of United States Patent No. 5,270,057 under 35 U.S.C. §156, together with a duplicate of the papers thereof, certified as such, and three courtesy copies of the application. Also submitted are: (1) a power of attorney, (2) a declaration regarding the application for patent term extension and (3) copy of request for recordation of change of name.

The Commissioner is hereby authorized to charge $1,120.00 for receiving and acting on this application and any additional fees which may be required, or credit any overpayment, to Deposit Account No. 02-2334. Two duplicates of this paper are enclosed.

Respectfully submitted,



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Dated: 11/24/97

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE: :

U.S. PATENT NO. 5,270,057 :

ISSUED: December 14, 1993 :

TO: Andreas L.J. de Meere and Marinus A. De Ruiter :

FOR: STABILIZED GONADOTROPIN CONTAINING PREPARATIONS :

FROM: SERIAL NO. 914,227 :

OF: July 3, 1992 :

APPLICATION FOR EXTENSION OF
THE TERM OF UNITED STATES PATENT
NO. 5,270,057 UNDER 35 U.S.C. §156

Assistant Commissioner for Patents
Box Patent Extension
Washington, D.C. 20231

Sir:

Your Applicant, Akzo Nobel N.V. (hereafter "Akzo"), a corporation duly organized and existing under the laws of The Netherlands and having a place of business at Arnhem, The Netherlands, represents that it is the owner of the entire right, title and interest in and to Letters of Patent of the United States No. 5,270,057 granted to Andreas L.J. de Meere and Marinus A. De Ruiter on the 14th day of December, 1993, for "STABILIZED GONADOTROPIN CONTAINING PREPARATIONS", by virtue of an assignment, recorded in the United States Patent and Trademark Office on the 20th day of March, 1991, at Reel 5645, Frame 0930, and

the change of name from Akzo N.V. to Akzo Nobel N.V., a copy of the Request for Recording of which is submitted herewith.

This patent is a continuation of application Serial Number 672,509, filed March 20, 1991 and now abandoned. Pursuant to the Uruguay Round Agreements Act (URAA), the term of this patent, which was a patent in force on June 8, 1995, became March 20, 2011 -- 20 years from the date of its original United States filing.

Your Applicant now hereby applies for an extension of the term of said United States patent of 193 days under 35 U.S.C. §156, based on the materials set forth herein and in the accompanying papers.

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(1) The product subject to a regulatory review period is Follistim™ (follitropin beta for injection). Follistim™ contains human follicle-stimulating hormone (recombinant hFSH), a glycoprotein that is manufactured by recombinant DNA technology. Follistim™ is further identified as follows:

Description

Follitropin beta has a dimeric structure containing two glycoprotein subunits. Both the 92 amino acid alpha-chain and the 111 amino acid beta-chain have complex heterogeneous structures arising from two N-linked oligosaccharide chains. Follitropin beta is synthesized in a Chinese hamster ovary (CHO) cell line that has been transfected with a plasmid containing the two subunit DNA sequences for encoding hFSH. The recombinant DNA expression and purification process results in a highly purified preparation with a consistent hFSH isoform profile and high specific activity. Follistim™ is presented as a sterile, freeze-dried cake, intended for subcutaneous or intramuscular administration after reconstitution with 0.45% sodium chloride injection, USP. Each vial of Follistim™ contains 75 IU or 150 IU of FSH activity plus 25.0 mg sucrose, NF; 7.35 mg sodium citrate dihydrate, USP; 0.10 mg polysorbate 20, NF; and hydrochloric acid, NF and/or sodium hydroxide, NF to adjust the pH in a sterile, lyophilized form.

Generic Name

follitropin beta for injection

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(2) Follistim™ (follitropin beta for injection) has been subject to regulatory review under Section 505 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. §355).

(3) On September 29, 1997 Follistim™ (follitropin beta for injection) received permission for commercial marketing or use under Section 505 of the Federal Food, Drug, and Cosmetic Act (21 U.S.C. §355).

(4) The active ingredient in Follistim™ (follitropin beta for injection) is recombinant hFSH. No hFSH product manufactured by recombinant DNA technology has been previously approved for commercial marketing or use under the Federal Food, Drug, and Cosmetic Act, the Public Health Service Act or the Virus-Serum-Toxin Act. Recombinant hFSH differs from natural hFSH since the oligosaccharide side-chains are different. Moreover, in addition to differing in structure, Follistim™ is a significant advance in its purity profile over previously available FSH products, which were extracted from urine, such that Follistim™ is a unique product eligible for patent extension. Prior to Follistim™, gonadotropin preparations were derived from the urine of postmenopausal women containing only FSH (urofollitropin), or both FSH and luteinizing hormone (LH) activity (human menopausal gonadotropin, hMG). While these prior products were cleared for marketing by FDA for treatment of infertility, use of these traditional urinary preparations is associated with the following disadvantages: a) low purity; b) no absolute source control; c) cumbersome urine collection; d) LH contamination in "pure" FSH preparations; e) risk of batch-to-batch inconsistency; f) and low specific activity. The recombinant DNA

technology which allows the production of Follistim™ has made it possible to produce virtually unlimited quantities of a formerly scarce biological product. Furthermore, in comparative studies with Follistim™ and a urinary FSH product in in-vitro fertilization, significantly more oocyctes have been retrieved, more embryos obtained and, as a result, more pregnancies were achieved with Follistim™. (*see* Out, Mannaerts, Driessen and Bennink (1996) "Recombinant follicle stimulating hormone (rFSH; Puregon) in assisted reproduction: More oocytes, more pregnancies. Results from five comparative studies." *Human Reproduction.*, Update 1996, Vol. 2, No. 2, pp. 162-171; *see also* Geurts, Peters, Bruggen, Boer and Out (1996) "Puregon (ORG 32489) - Recombinant Human Follicle-Stimulating Hormone." *Drugs of Today*, Volume 32, No. 3, pp.239-258; *see also* Out, Mannaerts, Driessen and Bennink (1995) "A prospective, randomized, assessor-blind, multicentre study comparing recombinant and urinary follicle stimulating hormone (Puregon versus Metrodin) in in-vitro fertilization." *Human Reproduction*, Volume 10, No. 10, pp.2534-2540.)

(5) This application is being submitted within the period permitted for its submission pursuant to 35 U.S.C. §156(d)(1). The last day on which this application could be submitted is November 28, 1997.

[remainder of page intentionally left blank]

(6) The patent for which an extension is being sought is identified as follows.

Inventors: Andreas L.J. de Meere

Marinus A. De Ruiter

Patent No.: 5,270,057

Title: STABILIZED GONADOTROPIN CONTAINING PREPARATIONS

Issued: December 14, 1993

Expires: March 20, 2011 (pursuant to URAA)

(7) A copy of United States Patent No. 5,270,057 the patent for which an extension is being sought, is attached hereto as EXHIBIT 1.

(8) No disclaimer, certificate of correction or reexamination certificate has issued for United States Patent No. 5,270,057. The first maintenance fee was paid on June 5, 1997.

[remainder of page intentionally left blank]

(9) United States Patent No. 5,270,057 claims the product subject to regulatory review. Claim 7 is specifically limited to a lyophilized composition that contains recombinant FSH: this composition is the product subject to regulatory review per se. The manner in which the claim reads on the product subject to regulatory review is as follows:

Claim 7 of U.S. 5,270,057 claims a compound of the general formula:

one part by weight of a FSH, said FSH being of recombinant source; and
200 to 10,000 parts by weight of at least one salt of an organic acid selected from the group consisting of salts of citric acid, tartaric acid, aspartic acid, isocitric acid, glutamic acid and mixtures thereof.

Therefore, Claim 7 and the broader claims read on Follistim™, the drug product comprising recombinant FSH subject to regulatory review.

[remainder of page intentionally left blank]

(10) The following relevant dates and information are provided to enable the Commissioner to determine pursuant to 35 U.S.C. §156(g) that, except for permission to market or use the product commercially, United States Patent No. 5,270,057 would be eligible for patent term extension under 35 U.S.C. §156:

(a) A New Drug Application ("NDA") under Section 505 of the Federal Food, Drug, and Cosmetic Act for Follistim™ (follitropin beta for injection) was filed on January 10, 1996, as NDA 20-582.

(b) NDA 20-582 was approved on September 29, 1997.

(11) A brief description of the significant activities undertaken by or for the applicant during the applicable regulatory review period with respect to the product subject to regulatory review and the significant dates applicable to such activities is attached hereto as EXHIBIT 2.

(12) (a) Applicant is of the opinion that United States Patent No. 5,270,057 is eligible for an extension under 35 U.S.C. §156, and the length of extension claimed is 193 days.

The requirements of 35 U.S.C. §156(a) and (c)(3) have been satisfied as follows:

(1) U.S. Patent No. 5,270,057 covers the drug product Follistim™ (follitropin beta for injection).

(2) U.S. Patent 5,270,057 is currently set to expire on March 20, 2011 (i.e., the term of the patent has not yet expired).

(3) The term of U.S. Patent No. 5,270,057 has never had an interim extension. The term has never been extended under 35 U.S.C. §156(e)(1).

(4) This application for extension is being submitted by Akzo, the owner of record of U.S. Patent No. 5,270,057, in accordance with the requirements of 35 U.S.C. §156(d).

(5) The product, Follistim™ (follitropin beta for injection), has been subject to a regulatory review period under section 505 of the Federal Food, Drug and Cosmetic Act before its commercial marketing or use, and permission for said commercial marketing or use is the first permitted commercial marketing or use under the Federal Food, Drug and Cosmetic Act.

(6) No patent has to this date been extended, nor have any other extensions been applied for, for the regulatory review period which forms the basis for this application for extension of the term of U.S. Patent No. 5,270,057.

(b) The length of extension of the term of U.S. Patent No. 5,270,057 of 193 days claimed by applicant was determined according to the provisions of 35 U.S.C.§156(d)(1) and (c)(3) as follows:

(1) U.S. Patent No. 5,270,057 was issued before the enactment of the URAA. Therefore, effective June 8, 1995, the term of U.S. Patent No. 5,270,057 was reset to expire on March 20, 2011. 35 U.S.C. §156(c) provides that a patent eligible for extension is to be extended under that provision by the time equal to the regulatory review period for the approved product, which period occurs after the date the patent is issued.

(2) If not for subsection (c)(3), U.S. Patent No. 5,270,057 would be eligible for extension of 628 days. This extension would be derived by calculating the regulatory review period. Therefore, but for subsection (c)(3), U.S. Patent No. 5,270,057 would be eligible to be extended to December 8, 2012.

(3) The exception in subsection (c)(3) applies to U.S. Patent No. 5,270,057, which operates to limit the extension to September 29, 2011, which is a period of 14 years from "the date on which the product involved receive[d] approval for commercial marketing or use." Therefore, under the relevant provisions of 35 U.S.C. §156, Applicant is entitled to an extension corresponding to the period from March 20, 2011 to September 29, 2011. This is 193 days, which is the length of the extension being claimed. Hence, Applicant is in compliance with 35 U.S.C.§156.

(13) Applicant acknowledges a duty to disclose to the Commissioner of Patents and Trademarks any information that is material to the determination of entitlement to the 193 day extension being sought to the term of United States Patent No. 5,270,057.

(14) Please charge the fee of $1,120.00 for receiving and acting on this application for extension and any underpayments or credit any overpayments to Deposit Account No. 02-2334, for which duplicate copies are enclosed.

(15) Please address all inquiries and correspondence relating to this application for patent term extension to:

William M. Blackstone

Akzo Nobel

1300 Piccard Drive, Suite 206

Rockville, MD 20850-4373

(301) 948-7400

(16) A duplicate of these application papers, certified as such, is enclosed herewith.

(17) A declaration as set forth in 37 C.F.R. §§1.740(a) and 1.740(b) is enclosed herewith.

William M. Blackstone
Attorney for Applicant
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Akzo Nobel NV
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Dated: 11/24/97

REFERENCES

Human Reproduction Update 1996, Vol. 2, No. 2 pp. 162–171

Recombinant follicle stimulating hormone (rFSH; Puregon) in assisted reproduction: More oocytes, more pregnancies. Results from five comparative studies

Henk J.Out[1], Bernadette M.J.L.Mannaerts, Stefan G.A.J.Driessen and Herjan J.T.Coelingh Bennink

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The clinical assessment of recombinant follicle stimulating hormone (rFSH; Puregon) in assisted reproduction technologies such as in-vitro fertilization (IVF) has probably been the most extensive clinical trial programme ever performed for the evaluation of a new fertility drug. It started with a pilot study to evaluate the potential of rFSH to stimulate the ovaries in the absence of luteinizing hormone (LH) using various gonadotrophin-releasing hormone (GnRH) agonists. After it became clear that FSH-induced steroidogenesis was not jeopardized even after severe pituitary suppression, comparisons between rFSH and urinary FSH or human menopausal gonadotrophins were made using different GnRH agonists or no agonists at all. In addition, the effects of the route of administration (s.c. or i.m.) were assessed. The study with the strongest statistical power to truly assess clinically relevant differences between rFSH and urinary FSH included ~1000 patient cycles. It indicated that after rFSH treatment, significantly more oocytes were retrieved, more embryos obtained and, as a result, more pregnancies achieved when the results of the cryoprogramme were included.

Key words: IVF/recombinant FSH/urinary FSH

Introduction

Assisted reproduction technologies such as in-vitro fertilization (IVF), gamete intra-Fallopian transfer, zygote intra-Fallopian transfer and intracytoplasmic sperm injection all require ovarian stimulation to increase the number of female gametes, and hence the chances for a successful treatment outcome (Palermo *et al.*, 1992; Healy *et al.*, 1994). Currently, this is achieved by gonadotrophin treatment with human menopausal gonadotrophins (HMG) or urinary follicle stimulating hormone (FSH). In addition, the use of gonadotrophin-releasing hormone agonists (GnRHa) is now routine in most IVF clinics, because it enables the adequate timing of oocyte recovery without the potential interference of premature luteinizing hormone (LH) surges (Tarlatzis *et al.*, 1994). Recent meta-analyses indicate that the use of GnRHa in a long protocol and of FSH-only preparations yields better results in IVF than short protocols and the administration of HMG respectively (Hughes *et al.*, 1992; Daya *et al.*, 1995).

Disadvantages of urinary gonadotrophins

Until recently, gonadotrophins for medical use were all of a urinary origin. This source implies a number of disadvantages, including low purity (<5%), no absolute source control, a cumbersome collection of urine, some LH contamination even in 'pure' FSH and a low specific activity.

[1]To whom correspondence should be addressed. Phone: 31–412–662816; Fax: 31–412–662530

The recently available rFSH is nearly 100% pure without LH contamination, and displays a better batch-to-batch consistency compared with the urinary products because of the carefully controlled manufacturing conditions (Loumaye *et al.*, 1995).

How to assess comparative safety and efficacy profiles of gonadotrophins

Studies addressing the safety and efficacy of gonadotrophins should be set up prospectively including a randomized design. Ideally, the design should also be double-blind. A sufficient number of patients should be included to provide the trial with an adequate power, generally requiring the cooperation of multiple centres. A recent overview of controlled randomized trials in the infertility field indicated that those using a randomized methodology, including studies comparing the effectiveness of gonadotrophins, were relatively few in comparison with other branches of medicine (Vandekerckhove *et al.*, 1993). In addition, most studies have suffered from unrealistically small sample sizes, the inappropriate use of crossover design or pseudo-randomization (Vandekerckhove *et al.*, 1993). Relevant endpoints of comparative trials assessing the properties of gonadotrophins in assisted reproduction are given in Table I.

Table I. Endpoints of comparative clinical trials in in-vitro fertilization assessing properties of gonadotrophins

Endpoints	Property
Efficacy endpoints	No. of follicles
	No. of oocytes
	No. of transferable embryos
	No. of pregnancies
Efficiency endpoints	Total dose (IU) administered
	Treatment length (days)
Safety endpoints	Incidence of ovarian hyperstimulation syndrome
	Incidence of multiple gestation

The best endpoint of the efficacy of, for instance, an FSH preparation is the number of large follicles recruited after a fixed period of time using a fixed dose in pituitary-suppressed women. However, in IVF, treatment regimens usually include possibilities of adapting the dose depending on the ovarian response, as assessed by ultrasound and serum oestradiol concentrations. In addition, in multicentre studies with sometimes two or more persons involved in ultrasound scanning, follicle size measurements are not very exact because of interobserver variability (Zador *et al.*, 1988); for that reason they are less suitable as an efficacy endpoint. In addition, comparisons between gonadotrophins based on parameters on the day of human chorionic gonadotrophin (HCG) administration will have to take into account the fact that these variables not only reflect the effects of gonadotrophin stimulation itself but also the local ovarian stimulation strategy to reach a predefined criterion, e.g. the number and size of follicles and/or the oestradiol concentration at which HCG is administered. For that reason, efficiency parameters are also important, defined as the total dose (IU) administered and/or the treatment duration.

In multicentre IVF studies comparing gonadotrophins, the most feasible primary efficacy endpoint is therefore the number of oocytes retrieved, because this is the direct result and goal of ovarian stimulation and the parameter that is most easily assessed. Pregnancy rates are less suitable because they rather depend on the number of embryos replaced, which is usually fixed at a certain number. Any surplus of embryos obtained as a result of a higher number of follicles recruited and oocytes retrieved (because of a better performance of the gonadotrophin studied) is therefore not apparent and not reflected in the pregnancy rates directly following the stimulation cycle, unless the gonadotrophin used has a direct effect on the oocyte and subsequently on embryo quality. Therefore it seems that pregnancy rates should include the results of frozen–thawed embryo replacements that are the direct (quantitative) result of the preceding gonadotrophin stimulation cycle (Queenan *et al.*, 1995).

Take-home baby rates, although important in view of the ultimate goal of infertility treatment, should be considered as a less adequate endpoint of gonadotrophin action because other (e.g. obstetric) variables will influence these rates.

Traditionally, safety endpoints of gonadotrophins include the incidence of ovarian hyperstimulation syndrome (OHSS) and multiple gestation, although in IVF the latter is the result of the number of embryos replaced rather than a consequence of the gonadotrophin *per se* (Hull, 1995).

To reliably assess the safety and efficacy of rFSH, (Puregon; Organon, Oss, The Netherlands) a large-scale clinical trial programme in IVF has been organized, including studies that were all prospective and randomized; most of them were multicentre investigations. Comparative drugs involved HMG and urinary FSH, and various GnRHa were applied (Table II).

For technical reasons, these studies were not double-blind because rFSH was provided in vials and urinary FSH in ampoules. Instead, the studies were conducted assessor-blind, implying that the person making the decisions on treatment regimens was not aware of the medication used.

Table II. Clinical trial programme of recombinant follicle stimulating hormone (FSH) in in-vitro fertilization

First author	Patients treated (*n*)	Centres (*n*)	Comparative gonadotrophin	Gonadotrophin-releasing hormone agonist
Devroey *et al.* (1994)	51	1	None	Various[a]
Out *et al.* (1995a)	981	18	Urinary FSH	Buserelin
Hedon *et al.* (1995)	90	6	Urinary FSH	Triptorelin
Jansen and Van Os (1996)	89	1	Human menopausal gonadotrophin	None
Out *et al.* (1995b)	195	12	Puregon s.c. versus i.m.	Buserelin

[a]See Table III.

Table III. Median serum luteinizing hormone (LH) and oestradiol concentrations in each group at baseline starting recombinant follicle stimulating hormone (rFSH) treatment and on the day of human chorionic gonadotrophin (HCG) administration

		LH (IU/l)	Oestradiol (pmol/l)
Group I: rFSH alone	Baseline	6.7	143
	Day HCG	5.1	4042
Group II: buserelin short	Baseline	4.9	121
	Day HCG	2.3	6971
Group III: buserelin long	Baseline	3.2	121
	Day HCG	1.3	6509
Group IV: triptorelin i.m.	Baseline	2.8	99
	Day HCG	1.2	6490
Group V: triptorelin s.c.	Baseline	2.4	88
	Day HCG	1.6	5620

The statistical analysis took into account centre effects in multicentre studies by weighting the SE of the treatment difference of the two comparative drugs. The higher the SE (e.g. because of a small number of subjects recruited), the smaller the weight of that centre in the analysis (Whitehead and Whitehead, 1991). Confidence intervals of 95% of the estimated treatment difference were calculated to assess whether clinically relevant differences were detected.

All analyses were performed on an intent-to-treat basis, including all subjects who received at least one dose of FSH. The main advantages of this rule are that more patients are available for the final analysis of efficacy and that it more closely reflects how physicians evaluate a therapeutic agent in the clinical setting, outside an experimental control.

Data management included monthly on-site monitoring with source verification, and extensive validation and verification procedures (remote data entry, data queries, double data entry, etc.). All trials were conducted according to Good Clinical Practice standards (CPMP Working Party, 1990).

The FSH content of the ampoules was standardized at 75 IU in-vivo bioactivity per ampoule, using the Steelman–Pohley assay (Steelman and Pohley, 1953) as required by the pharmacopoeia (Council of Europe, 1986).

Pilot efficacy study in IVF

In an open pilot efficacy study, the effects of rFSH in assisted reproduction were investigated for the first time using various down-regulation protocols to assess whether the administration of an FSH-only preparation in these circumstances would be sufficient for follicular development and adequate steroidogenesis (Devroey *et al.*, 1994).

In total, 51 infertile women were treated with rFSH alone (group I), with rFSH in conjunction with buserelin intranasal spray 4 ×150 μg daily in a short (group II) or long protocol (group III), or by using triptorelin in a long protocol, giving a single dose of 3.75 mg i.m. (group IV) or daily s.c. injections of 200 μg (group V). In all women, treatment with rFSH resulted in multiple follicular growth and rises in serum inhibin and oestradiol concentrations. The latter indicated that the amount of remaining LH was sufficient to support FSH-induced oestrogen biosynthesis (see Table III).

The median number of ampoules and treatment days required in the various treatment groups varied from 21 to 36 ampoules and from 7 to 14 days respectively. The median number of oocytes per group ranged from nine to 11. In all, 43 couples had an embryo transfer and the median number of embryos replaced in each was three per transfer. Eight ongoing pregnancies were achieved, resulting in the birth of nine healthy children (Devroey *et al.*, 1992a,b, 1993).

rFSH versus urinary FSH

Intranasal buserelin in a long protocol

The pivotal trial in the programme encompassed 1000 cycles (Out *et al.*, 1995a). This study was set up to detect even small differences between both groups. The primary endpoints, as defined prior to starting the study, were the number of oocytes retrieved and the ongoing pregnancy rate, defined as the presence of a vital pregnancy at least 12 weeks after embryo transfer. With a randomization in a 3:2 ratio between rFSH and urinary FSH, 80% power and a two-sided significance level of 5%, a difference of 1.2 oocytes retrieved (assuming SD = 6) and 6% in the pregnancy rates could be detected.

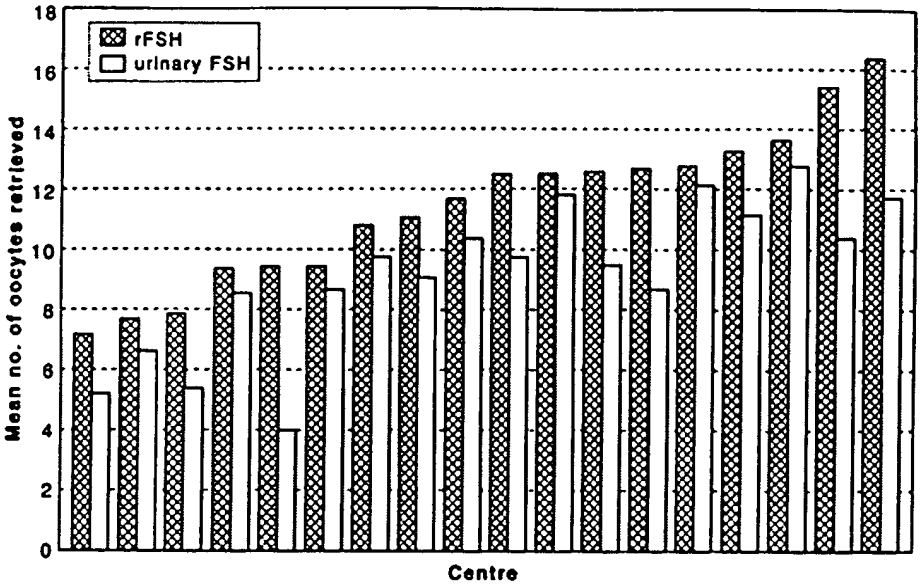

Figure 1. Number of oocytes retrieved in 18 different centres. FSH = follicle stimulating hormone.

Table IV. Mean results of recombinant follicle stimulating hormone (rFSH) versus urinary FSH using buserelin in a long protocol

Parameter	rFSH	Urinary FSH	95% confidence interval of treatment difference	*P* value
No. of subjects treated	585	396		
Total no. of oocytes retrieved	10.8	9.0	1.2 to 2.6	<0.0001
No. of mature oocytes retrieved	8.6	6.8	1.1 to 2.4	<0.0001
Total FSH dose (IU)	2138	2385	−338 to −158	<0.0001
Duration of treatment (days)	10.7	11.3	−0.9 to −0.3	<0.0001
No. of follicles ≥17 mm in diameter	4.6	4.4	−0.0 to 0.5	0.09
No. of follicles ≥15 mm in diameter	7.5	6.7	0.4 to 1.2	0.0002
Maximum serum oestradiol concentration (pmol/l)	6084	5179	494 to 1317	<0.0001
No. of high quality embryos	3.1	2.6	0.2 to 0.8	0.003
Ongoing pregnancy rate per attempt (%)	22.2	18.2	−1.1 to 9.0	0.13
Ongoing pregnancy rate per transfer (%)	26.0	22.0	−1.9 to 9.8	0.19
Ongoing pregnancy rate per attempt, including frozen embryo cycles (%)	25.6	20.4	0.0 to 10.6	0.05

The trial was designed as a prospective, randomized, assessor-blind, multicentre study. In all, 18 centres from 11 European countries participated. Selection criteria were age between 18 and 39 years, good physical health, normal weight, at least 1 year of infertility, no male factor, no endocrine abnormalities and normal regular ovulatory cycles. The protocol included: intranasal buserelin down-regulation in a long protocol; 150 or 225 IU for the first 4 days, after which the dose was adapted according to ovarian response; HCG administration when at least three follicles ≥17 mm in diameter were seen; and a maximum replacement per transfer of three embryos. Embryo morphology was assessed using a scoring system as published previously (categories I–IV; Staessen *et al.*, 1989). Embryos with types I and II classification were considered to be of high quality. The results are given in Table IV.

The main efficacy parameter, i.e. the number of oocytes retrieved, was consistently higher after rFSH treatment in all 18 participating centres (Figure 1). The fact that no significant difference was found in the number of follicles ≥17 mm in diameter on the day of HCG administration confirmed that the overall adherence to the protocol was adequate, because at least three follicles of that size had to be present before administering HCG. Follicular growth, as measured by the number of large (≥15 mm in diameter) follicles, started to diverge between rFSH and urinary FSH after ~5 days of

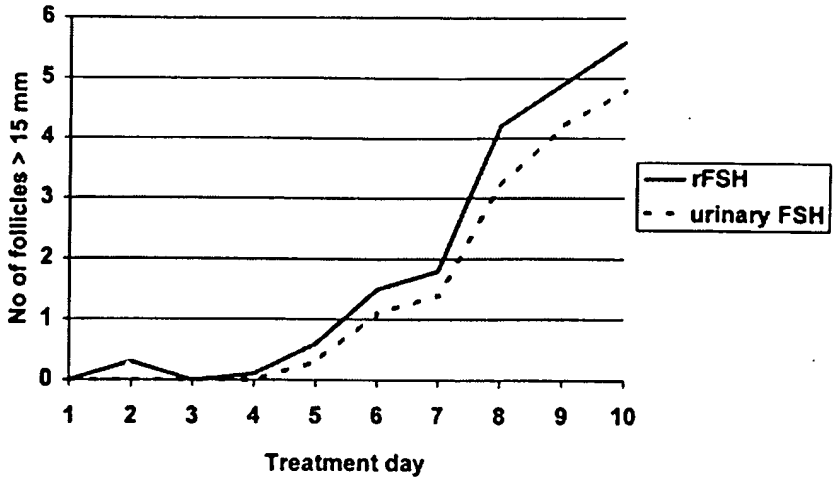

Figure 2. Number of follicles ≥15 mm in diameter related to the follicle stimulating hormone (FSH) treatment day.

treatment (Figure 2). Ongoing pregnancy rates, including frozen–thawed embryo replacements in subsequent natural cycles, were significantly in favour of rFSH ($P = 0.05$).

Cycle cancellations were seen in 152 subjects who started FSH treatment (rFSH, $n = 85$, 14.5%; urinary FSH, $n = 67$, 16.9%; not significantly different). A low ovarian response as the reason for cancellation was reported in 27 subjects in the rFSH group (4.6%) and in 30 subjects in the urinary FSH group (7.6%, $P = 0.036$, Fisher's exact test, not adjusted for the centre).

The incidence of OHSS leading to hospitalization was seen in 19 out of 585 rFSH-treated subjects (3.2%) versus eight out of 396 urinary FSH-treated subjects (2.0%), which was not significantly different.

In conclusion, this study, which was the largest prospective randomized clinical trial ever performed in IVF, demonstrated a significantly higher number of oocytes, embryos and ongoing pregnancies (efficacy endpoints) using a lower total dose during a shorter treatment period (efficiency endpoints) with a similar incidence of OHSS (safety endpoint) for rFSH when compared with urinary FSH.

Ongoing pregnancy rates with fresh and frozen embryos given in the initial report of the trial (Out *et al.*, 1995) are shown in Table V. Each individual comparison by cycle showed a numerical superiority in pregnancies with rFSH. For purposes of statistical analysis, the proportions in Table V were represented as a $3 \times 2 \times 2$ contingency table and the resulting frequencies analysed using log-linear modelling (GLM) methods on the algorithm GENSTAT. Individual comparisons failed to reach statistical significance, but the pooled analysis gave a significance probability of <5%, giving evidence of the superiority of rFSH.

S.c. triptorelin in a long protocol

In a multicentre ($n = 6$) French study comparing rFSH and urinary FSH with the daily s.c. administration of 100 µg triptorelin in a long protocol, 90 subjects were randomized and treated in a 3:2 ratio between rFSH and urinary FSH (Hedon *et al.*, 1995). The protocol was similar as described above. The results are given in Table VI.

Median maximum oestradiol concentrations were 7551 and 5514 pmol/l in the rFSH and urinary FSH groups respectively. None of the between-group differences was statistically significant. There were three cases of OHSS in the rFSH group, of which two had an ongoing pregnancy. No OHSS was seen in the urinary FSH group.

Table V. Pregnancies arising after the use of rFSH and urinary FSH

	Cycle 1: Fresh embryos	Cycle 2: First frozen embryos	Cycle 3: Second frozen embryos
rFSH	0.222 (130/585)	0.145 (17/117)	0.192 (5/26)
urinary FSH	0.178 (72/396)	0.068 (5/73)	0.113 (2/15)
Ratio	1.25	2.13	1.70

Table VI. Mean results of recombinant follicle stimulating hormone (rFSH) versus urinary FSH using triptorelin in a long protocol

Parameter	rFSH	Urinary FSH	95% confidence interval of treatment difference
No. of subjects treated	57	33	
Total no. of oocytes retrieved	9.7	8.9	−1.7 to 3.2
No. of mature oocytes retrieved	8.1	6.9	−1.1 to 3.4
Total FSH dose (IU)	2265	2213	−240 to 330
Duration of treatment (days)	10.2	10.3	−0.8 to 0.7
No. of follicles ≥17 mm in diameter	5.4	5.5	−1.2 to 0.9
No. of follicles ≥15 mm in diameter	7.3	7.2	−1.2 to 1.5
No. of high quality embryos	3.7	4.0	−1.7 to 1.1
Ongoing pregnancy rate per attempt (%)	30.2	17.4	−6.4 to 31.9
Ongoing pregnancy rate per transfer (%)	34.0	18.8	−5.5 to 35.9

Table VII. Mean results of recombinant follicle stimulating hormone (rFSH) versus human menopausal gonadotrophin (HMG) without using a gonadotrophin-releasing hormone agonist (Jansen and Van Os, 1996)

Parameter	rFSH	HMG	95% confidence interval of treatment difference
No. of subjects treated	54	35	
Total no. of oocytes retrieved	11.2	8.3	−1.1 to 6.8
No. of mature oocytes retrieved	10.6	7.5	−0.7 to 6.9
Total FSH dose (IU)	1410	1365	−53 to 143
Duration of treatment (days)	6.2	6.0	−0.2 to 0.6
No. of follicles ≥15 mm in diameter	5.5	5.4	−1.3 to 1.5
Maximum oestradiol concentrations (pmol/l)	3889	3145	−365 to 1855
No. of high quality embryos	3.1	3.0	−1.8 to 2.0
Ongoing pregnancy rate per attempt (%)	22.2	17.1	−12.1 to 22.2
Ongoing pregnancy rate per transfer (%)	30.8	22.2	−13.4 to 30.5

rFSH versus HMG

In a randomized single-centre study, rFSH and HMG were compared in non-pituitary-suppressed women; 89 women were treated. Subjects received an i.m. gonadotrophin injection of 150–225 IU/day during the first 4 days of treatment, which started on cycle day 3. The further protocol was similar to that discussed previously, except for the criterion to give HCG: to prevent premature luteinization, HCG was already administered when at least two follicles ≥15 mm in diameter were seen. The results are given in Table VII.

rFSH compared favourably with HMG with respect to the main efficacy parameters (i.e. the number of oocytes retrieved, 11.2 versus 8.3, and the ongoing pregnancy rates per attempt and per transfer, 22.2 versus 17.1 and 30.8 versus 22.2% respectively). However, none of the differences in this study was significant.

rFSH s.c. versus i.m.

In a randomized study with a similar design to the large IVF study described earlier (Out *et al.*, 1995a), the efficacy and local side-effects of rFSH administered either s.c. or i.m. were investigated in 118 and 77 subjects respectively who received study medication (Out *et al.*, 1995b). Local side-effects were assessed daily by the patients, focusing on the presence (mild, moderate, severe) of swelling, itching, redness, pain and bruising at the injection site. The results are given in Table VIII.

There were no significant differences in efficacy, efficiency and overall safety endpoints. The only significant difference detected was the occurrence of bruising, which was seen more often ($P = 0.019$) in the s.c. group. This was attributed to the more superficial injection of the s.c. route, enabling better inspection of the occurrence of bruising compared with the i.m. route.

Table VIII. Mean results of recombinant follicle stimulating hormone (rFSH) s.c. versus i.m. using buserelin in a long protocol

Parameter	rFSH s.c.	rFSH i.m.	95% confidence interval of treatment difference[a]
No. of subjects treated	118	77	
Total no. of oocytes retrieved	10.4	9.8	−1.0 to 2.2
Total FSH dose (IU) administered	2115	2235	−270 to 38
Duration of treatment (days)	9.7	9.9	−0.6 to 0.2
Ongoing pregnancy rate per attempt (%)	26.1	27.1	−13.6 to 11.6
Ongoing pregnancy rate per transfer (%)	29.3	30.1	−14.6 to 12.9
Local side-effects[b]			
Overall (%)	68.6	63.6	−8.8 to 18.4
Bruising (%)[c]	54.2	37.7	2.6 to 29.7
Pain (%)	28.0	31.2	−16.2 to 8.2
Redness (%)	16.1	13.0	−6.5 to 14.2
Swelling (%)	5.9	7.8	−9.4 to 5.4
Itching (%)	3.4	6.5	−9.3 to 3.0

[a]Based on means adjusted for centre.
[b]Defined as the presence of at least one treatment day with symptom(s).
[c]$P = 0.019$.

Discussion

The large IVF study unambiguously demonstrated that rFSH (Puregon) had a better efficacy profile than urinary FSH: more oocytes were retrieved, more high quality embryos obtained and, ultimately, after replacement of the frozen–thawed embryos, a higher ongoing pregnancy rate was seen (Out *et al.*, 1995a). A higher activity of rFSH was also suggested by the significantly lower cancellation rate for low response compared with urinary FSH. The two supportive studies demonstrated the same trend towards a higher number of oocytes retrieved and the ongoing pregnancy rate, although these differences were not significantly different probably because of the smaller sample size (Hedon *et al.*, 1995; Jansen and Van Os, 1996). This emphasizes the need for large studies comprising many patients so as to reliably detect subtle differences between gonadotrophin preparations. Oestradiol concentrations at the day of HCG administration, which can be considered as a sensitive measure of follicle size and number, were higher after rFSH treatment in all studies, again indicating that rFSH has a higher bioactivity than urinary FSH or HMG.

The fact that ongoing pregnancy rates were consistently higher (although not significantly) immediately following embryo transfer in the stimulation cycle suggests that rFSH might have direct beneficial effects on oocyte and embryo quality, leading to higher implantation rates. In addition, the availability of a surplus of embryos improves the possibilities for selection by the embryologist who will replace the best embryos obtained.

Calculation of the cumulative ongoing pregnancy rates including frozen–thawed embryo replacements is the best way to assess the efficacy of an IVF stimulation cycle (Toner *et al.*, 1991; Wang *et al.*, 1994; Queenan *et al.*, 1995). For rFSH, it was demonstrated that these rates were higher than for urinary FSH. This might be a result of the availability of better quality embryos after rFSH treatment because an equal number of embryos in these natural cycles was replaced in both groups, or the more obvious reason that merely the availability of more embryos will ultimately lead to more pregnancies. Because the reported pregnancy rates do not include the replacements of all embryos available, differences in the projected ongoing pregnancy rate after using all available embryos will be even more than the 6% difference reported in favour of rFSH.

The reasons for the higher effectiveness of rFSH are not known. Possible explanations include the content of the more basic isohormones in rFSH, differences in pharmaceutical formulation, the presence of FSH-inhibiting substances in urinary FSH and small differences at the carbohydrate level.

Future research is needed to elucidate the influence of these factors on the better performance of rFSH. In fact, its higher activity is surprising in view of the equal contents of ampoules of both rFSH and urinary FSH, namely 75 IU in-vivo bioactivity, as determined by the in-vivo Steelman–Pohley rat assay (Steelman and Pohley, 1953). If this

in-vivo bioassay used to standardize the bioactivity per ampoule was predictive for efficacy in IVF, by definition no difference could have been detected in clinical trials. Apparently, this bioassay, which is required as per pharmacopoeia (Council of Europe, 1986), is not valid for a proper clinically relevant assessment of the bioactivity of an FSH preparation. This is not surprising because in-vivo bioactivity is measured as the weight augmentation of ovaries from rats treated with FSH and a surplus of HCG in a comparative way with an international reference preparation. Microscopical examination of these ovaries reveals many luteinized unruptured follicles that contain large volumes of follicular fluid which contribute disproportionately to the weight augmentation (unpublished observations).

It is unlikely that the known relatively large inter- and intra-assay variations of the Steelman–Pohley assay, leading to coincidental differences in the number of international units per ampoule, account for the differences found. Apart from the fact that pharmaceutical companies can easily overcome this problem by multiple analyses and careful standardizing of their procedures, a direct linear relationship between the mean daily dose or the total dose given to reach the criterion to administer HCG and the number of oocytes retrieved could not be confirmed (Out *et al.*, 1995a).

Clinical trials have also been conducted comparing urinary FSH with another rFSH preparation (Gonal-F; Ares-Serono, Geneva, Switzerland). One completed study has been published assessing the effectiveness of this rFSH in IVF (Recombinant Human FSH Study Group, 1995). Table IX summarizes the main results of that study.

Unfortunately, confidence intervals were not given, but the small sample size obviously implies that clinically relevant differences could not be detected in this study. Despite this, these data strongly suggest that differences between the rFSH preparations Puregon and Gonal-F exist, given the significantly lower oestradiol concentrations and number of follicles ≥14 mm in diameter on the day of HCG administration compared with urinary FSH, which is completely i contrast to the results obtained in the comparison of Puregc and urinary FSH. This may be expected because of the com plexity of the recombinant DNA preparations involved. Th different natures of the two rFSH preparations are confirme by the fact that the regulatory authorities have issued tw distinct International Non-proprietary Names (INN), i.e follitrophin alpha (Gonal-F) and follitrophin beta (Puregon to emphasize the fact that both compounds should be con sidered as distinct new active substances. Direct compari sons between both preparations will ultimately demonstrat whether these compounds have a different clinical efficacy

Efficiency endpoints, defined as the treatment duratio and the total dose administered, were significantly advan tageous for rFSH. This indicates a double benefit: not onl is the number of oocytes retrieved higher, but pre-ovula tory conditions are reached using fewer ampoules in a shorter treatment period. Obviously, this means improve convenience for the patients and the fertility specialists.

No significant differences between rFSH and urinary FSH or HMG were found with respect to the incidence of the mos important side-effect, OHSS. In the large IVF study (Out *et al.*, 1995a), severe OHSS (hospitalization) was seen in 3.2% of women after rFSH treatment versus 2.0% in the urinary FSH group. However, because risk factors for the development of OHSS, such as a high number of follicles and high oestradiol concentrations, were seen more often in the rFSH group, adequate monitoring of the ovarian response remains essential to prevent this complication.

No antibodies against FSH or Chinese hamster ovary cell-derived proteins were detected. In addition, no clinically relevant changes in the biochemical or haematological parameters were found, nor significant trends in vital signs, as assessed before and after treatment. The s.c. route enabling self-administration was well tolerated, as assessed prospectively in comparison with the i.m. route.

Table IX. Results of a multicentre clinical trial in in-vitro fertilization and embryo transfer comparing another recombinant follicle stimulating hormone (rFSH) preparation (Gonal-F) with urinary FSH

Parameter	Gonal-F	Urinary FSH	*P* value
No. of subjects treated	60	63	
Total no. of oocytes retrieved	9.3	10.7	NS
Total FSH dose (IU)	2270	2095	NS
Duration of treatment (days)	9.9	9.4	NS
No. of follicles ≥14 mm in diameter	7.8	9.2	0.037
Oestradiol concentrations at day of HCG administration (pmol/l)	5781	8046	0.0007
Delivery of one or more live infants (%)	15	13	NS

HCG = human chorionic gonadotrophin; NS = not significant.

Conclusion

An rFSH preparation (Puregon) has indisputably demonstrated a better clinical performance than urinary FSH in assisted reproduction, as assessed in the largest prospective randomized IVF trial ever performed. This was ultimately shown by an increased ongoing pregnancy rate when frozen–thawed embryo replacements were included. No significant differences in the safety profiles were seen, which altogether makes rFSH the first choice of treatment in infertile couples who need gonadotrophin ovarian stimulation.

Acknowledgements

The following investigators were involved in the Puregon trials mentioned in this article. Belgium: Centre for Reproductive Medicine, Academic Hospital Free University, Brussels — P.Devroey, A.van Steirteghem, J.Smitz. Denmark: Rikshospitalet, Department of Gynaecology, Fertility Clinic, Copenhagen — P.Hornnes. Finland: Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, Helsinki — A.Tiitinen, N.Simberg, M.Tulppala, M.T.Seppälä. France: Centre de Procréation Médicalement Assistée, 80054 Amiens — B.Camier; Centre Hospitalier Intercommunal Jean Rostand, 923311 Sévres — J.Cohen, J.Belaïsch-Allart, L.Nicolle; Centre Hospitalier et Universitaire de Montpellier, 34295 Montpellier — B.Hedon, C.Humeau, L.Lafont; Hôpital Jean Verdier, 93143 Bondy — J.N.Hugues, I.Cedrin Durnerin; Hotel Dieu, 40000 Nantes — P.Lopes, B.Charbonnel, P.Barrière, F.X.Laurent; Clinique Universitaire Baudelocque, 75014 Paris — J.R.Zorn, I.Matheron. Germany: Universitäts-Frauenklinik, Bonn — K.Diedrich, C.Diedrich; Universitäts-Frauenklinik, Erlangen — L.Wildt, E.Siebzehnrübl, B.Munzer. Greece: Infertility and IVF Centre 'Geniki Cliniki', Thessaloniki — B.C.Tarlatzis, J.Bontis, S.Lagos, H.Bili, D.Bakratsa, S.Mantalenakis. Ireland: Human Assisted Reproduction Ireland, RCSI Academic Department of Obstetrics and Gynaecology, Rotunda Hospital, Dublin — R.F.Harrison, U.Kondaveeti, B.Hennelly, A.Gordon, L.Drudy, E.Cottell, A.MacMahon. Israel: Department of Obstetrics and Gynaecology, Kaplan Hospital, Rehovoth — Z.Shoham, A.Barash, P.Golan, I.Segal, L.Sindel, V.Insler. The Netherlands: Department of Obstetrics and Gynaecology, Diaconessenhuis, Voorburg — C.Jansen, L.van Os. Norway: Kvinnekliniken Rikshopitalet, Oslo — T.Åbyholm, T.Tanbo, L.Henriksen; Kvinneklinikken Regionsykehuset i Trondheim, Trondheim — J.Kahn, A.Sunde, L.Reinertsen. Spain: Servicio de Reproducción Humana, Instituto Dexeus, Barcelona — P.Barri, P.Cabrero, M.Torello. Sweden: Kvinnokliniken, Sahlgrenska sjukhuset, Göteborg — L.Hamberger, L.Nilsson, C.Bergh, A.Strandell, B.Josefsson; Department of Obstetrics and Gynaecology, University of Lund, Malmö — N.O.Sjöberg, L.Hägglund, F.Ploman. UK: Department of Obstetrics and Gynaecology, Guy's Hospital, London — R.G.Forman, S.A.Adeaga; Regional IVF Unit, St Mary's Hospital, Manchester — B.Lieberman, P.Buck, P.Matson, F.Hamer, A.Ratcliffe, J.Bent, G.Horne; Department of Obstetric and Gynaecology, University of Wales College of Medicine, Cardiff — R.W.Shaw, S.Vine; The Assisted Conception Unit, Royal Infirmary of Edinburgh, Edinburgh — C.P.West, H.Hillier; Hammersmith Royal Postgraduate Medical School, Institute of Obstetrics and Gynaecology, London and IVF Clinic, Royal Masonic Hospital, London — R.M.L.Winston, N.Reddy, G.Atkinson, A.Huodono. The following persons from Organon contributed to the success of the Puregon clinical trial programme: A.Blum, B.de Blank, W.de Boer, H.Coelingh Bennink, P.Coudray, S.Driessen, F.Eikelboom, J.Geelen, U.Garrett-Jones, P.Geurts, B.vd Heijden, P.Hendrix, L.Jansen, P.Janssen, H.Joosten, E.Kleber, G.Lamond, R.de Leeuw, B.Mannaerts, M.Mälkönen, F.van Meel, A.de Meere, F.Metsers, J.Mulders, A.Nyberg, H.J.Out, R.Paulussen, C.vd Reydt, F.Rombout, A.Skrabanja, I.Solberg, C.Stavnstrup, S.Stuchbury, I.van de Veerdonk, R.Verbeeten, J.Verhoeven, J.vd Walle, P.Wright and many others.

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Received on February 23, 1996; accepted on March 4, 1996

DRUGS OF TODAY Vol. 32, No. 3, 1996, pp. 239-258

PUREGON® (ORG 32489) - RECOMBINANT HUMAN FOLLICLE-STIMULATING HORMONE

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CONTENTS

Summary

Follicle-stimulating hormone (FSH) is a complex glycoprotein essential for ovarian follicular development. It is used therapeutically in the treatment of human infertility, especially in anovulatory conditions and as hormonal pretreatment in assisted reproduction technologies such as *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Until recently, FSH could only be obtained from human urinary sources such as human menopausal gonadotropin (hMG), containing equal amounts of FSH and luteinizing hormone (LH), or as purified urinary FSH in which the LH content has been largely removed. The protein contents of most of these preparations contain more than 95% urinary contaminations. Furthermore, the use of urine implies limited sources, decreased flexibility and risks for batch-to-batch inconsistencies. In contrast, the availability of FSH manufactured by means of recombinant DNA technology is a great improvement. This review summarizes the characteristics of Puregon (Org 32489), a recombinant human FSH preparation, as established in an extensive research and development programme.

Introduction

Follicle-stimulating hormone (FSH) is produced and secreted by the anterior lobe of the pituitary. It is a glycoprotein hormone with a molecular mass of approximately 35 kD. FSH is indispensable in normal female and male gamete growth and maturation, as well as in gonadal steroid production. Consequently, deficient endogenous production of FSH can cause infertility, and studies have indicated that in developed countries about 15% of couples will experience infertility at some time during their reproductive lives (1). The already firmly established role for gonadotropins in the treatment of infertility is increasingly being confirmed and extended due to new technological developments, such as the use of assisted reproduction technologies.

Gonadotropin preparations are derived from the urine of postmenopausal women containing only FSH (urofollitropin), or both FSH and luteinizing hormone (LH) activity (human menopausal gonadotropin, hMG). They have been on the market for about 10 and 30 years, respectively. They have proved to be effective and safe in the treatment of female as well as male infertility. However, use of traditional urinary preparations is associated with a number of disadvantages, including low purity, no absolute source control, cumbersome urine collection, LH contamination in 'pure' FSH preparations, risk of batch-to-batch inconsistency, and low specific activity. Recently, recombinant DNA technology has made it possible to produce unlimited quantities of formerly scarce biological products. This technology was used to develop recombinant human FSH (hFSH) by transfecting a Chinese hamster ovary (CHO) cell line with a plasmid containing the two subunit genes encoding FSH (2).

The recombinant hFSH preparation Puregon (code name Org 32489), produced by Organon, is almost identical to natural hFSH. Structural and conformational analyses showed that the amino acid sequence and structure are indistinguishable from those of natural hFSH. In addition, the oligosaccharide side-chains are very similar to those reported for natural hFSH. The small differences do not affect the degree of charge heterogeneity, receptor binding affinity, or the *in vitro* and *in vivo* bioactivities of Puregon as compared to natural hFSH. Consequently, Puregon is expected to be safe and effective in ovarian stimulation in anovulatory infertility, in controlled ovarian hyperstimulation in medically assisted reproduction, and probably also in treatment of infertility associated with male hypogonadotropic hypogonadism.

Chemical and Pharmaceutical Information

Structural formula

FSH belongs to a family of structurally related glycoproteins including LH, chorionic gonadotropin (CG) and thyroid-stimulating hormone (TSH). Each protein is a heterodimer consisting of two non-covalently associated α- and β-subunits. Within a given animal species, the α-subunits of FSH, LH, CG and TSH are derived from a single gene (3, 4) and have identical amino acid sequences (5, 6). The β-subunits of each glycoprotein are derived from separate genes and differ in their amino acid sequences (3, 7, 8). It is the β-subunit which provides each hormone its specific biological function. The glycoprotein character of the proteins is expressed by the attachment of carbohydrate chains to the asparagine (Asn) amino acid residues on both subunits. The Asn-linked oligosaccharides on the α-subunit are attached at homologous locations, *i.e.*, Asn-52 and Asn-78, but they differ in structure for each gonadotropin. The Asn-linked oligosaccharides on the β-subunits differ in number and in location for each glycoprotein. The carbohydrate chains of the β-subunit of FSH are located at Asn-7 and Asn-24 and are also FSH-specific.

The assembly and posttranslational modification of glycoprotein subunits leading to functional heterodimers is a complex process which takes place in the endoplasmic reticulum (9, 10). Additional oligosaccharide modification, as well as sorting out of the hormones into secretory granules, occurs in the Golgi apparatus. This complex process results in the generation of a family of hormone isoforms which differ in carbohydrate composition. The oligosaccharide chains are a main determining factor for the biological activity of the hormone, since they i) influence receptor affinity, ii) are essential for the transduction of the biological signal into the target cell, and iii) have a strong impact on the plasma half-life of the hormone (6, 7, 11).

Molecular formula

The nucleotide sequences of the α- and β-subunits of recombinant hFSH were determined by sequencing of the FSH DNA constructs used for the transfection of the CHO host cell line. The sequences are identical to those reported for pituitary hFSH (4, 12). The primary structure of recombinant hFSH was determined after trypsin or lysC treatment of the isolated subunits followed by separation of the generated peptides by high pressure liquid chromatography (HPLC). There were no differences when compared to its natural counterpart. Recombinant hFSH contains $36 \pm 5\%$ (w/w) carbohydrates as determined by carbohydrate composition analysis

(13) using the Moore and Stein amino acid analysis method (14). Carbohydrate structure analysis by nuclear magnetic resonance spectroscopy on oligosaccharides which were enzymatically released from recombinant hFSH revealed that recombinant hFSH differs from natural hFSH in two aspects.

Firstly, the bisecting N-acetylglucosamine (GlcNAc) residues which are present in natural hFSH were not detected in recombinant hFSH, because CHO cells do not have the capability to incorporate those residues. Of the oligosaccharides, only 29% have an α1-6-linked fucose residue at the Asn-bound GlcNAc, whereas this proportion is about 50% in natural hFSH. Secondly, in some of the oligosaccharide fractions small amounts (<5%) of compounds were detected having one or more additional N-acetyllactosamine units (13).

All carbohydrate structures found in recombinant hFSH, however, represent moieties that have been described previously for natural human glycoproteins.

When analyzed by gel electrophoresis, recombinant hFSH migrated as a heterogeneous, diffuse protein band centered around an approximate mass of 45 kD. After dissociation of the intact molecule, the α- and β-subunits of recombinant hFSH displayed a collection of heterogeneous protein bands ranging in molecular mass from 19-33 kD. When analyzed under nondenaturing conditions by HPLC, recombinant hFSH elutes as a peak at 45 kD. These properties are identical to those for natural hFSH.

Isohormone profile

Natural gonadotropins display so-called microheterogeneity because they occur in various isoforms. This is due to differences in carbohydrate chain structure, especially in the degree of sialylation (11, 15-18). Isohormones can be separated by chromatofocusing or isoelectrofocusing techniques on the basis of differences in isoelectric points. Relatively acidic isohormones combine relatively low receptor binding affinity and intrinsic bioactivity with a long plasma residence time, whereas the relatively basic isoforms display relatively high receptor binding and intrinsic bioactivity together with a short plasma residence time (11, 19).

It was shown that pituitary hFSH could be separated into at least 20 isohormone fractions, which displayed 7 discrete levels of FSH receptor binding activities (20). After chromatofocusing, immunoreactive Puregon distributed over an isoelectric point range of 5.69-3.21, with a peak at 4.55 ± 0.12. This broad focusing range is similar to that of natural hFSH (11, 21) and urine-derived hFSH (Metrodin), the latter focusing between 5.58-3.08, with a peak at 4.29 ± 0.13. After isoelectric focusing, Puregon showed a multiple banding pattern focusing between pH 2.5 and 6.0. In a comparative study of Puregon and Metrodin it was shown that Puregon contains an about 2-fold lower proportion of relatively acidic isoforms (14.7% *vs.* 31.4%) and a 2-fold higher proportion of relatively basic isoforms (32.0% *vs.* 17.0%). Differences in isohormone composition, especially in the degree of sialylation, have a direct effect on the pharmacokinetics of gonadotropins and thus may influence their *in vivo* bioactivity. Isohormone fractions of Puregon obtained by chromatofocusing were all bioactive in the Sertoli cell aromatase bioassay (22).

Pharmaceutical formulation

Puregon consists of a sterile powder for injection which must be dissolved in a solvent for reconstitution prior to use (NaCl 0.45%). Puregon has four clinical dose strengths of 50, 75, 100 and 150 IU *in vivo* bioactivity recombinant hFSH. The strengths of 50,100 and 150 IU are provided in an ampoule as white to off-white freeze-dried spheres (lyospheres). The strength of 75 IU is provided in a vial as a lyophilized cake.

Preclinical Studies

Animal pharmacology

FSH exerts its biological effects via binding to specific high-affinity hormone receptors present in the target cell membrane. As a consequence of hormone-receptor complex formation, the synthesis of intracellular second messenger molecules such as cyclic AMP is stimulated, resulting in the stimulation of steroidogenesis and the expression of differentiation-related genes (23, 24).

1) FSH receptor binding

The receptor binding capacity of Puregon was examined in receptor displacement studies using calf testis membranes and ^{125}I-iodinated pituitary hFSH. Puregon inhibited the receptor binding of iodinated FSH in a dose-dependent manner. The displacement curves of Puregon and three reference preparations, IS 83/575 (an international pituitary-derived reference preparation for gonadotropins), Metrodin and Humegon, were parallel (25). This indicates comparable dose-dependencies for all preparations tested. The receptor binding affinity of Puregon was 2- to 3-fold higher than that of the three reference preparations when these preparations were added on the basis of their declared *in vivo* bioactivity as determined by the rat ovarian weight augmentation assay. When added in terms of immunoreactive FSH, the affinities of Puregon and Metrodin were similar (25), indicating that the *in vivo* bioactivity/immunoreactivity ratio (B/I) varied between preparations. The apparent discrepancy between

the receptor binding affinities using the declared *in vivo* bioactivity as described above is ascribed to isohormone profile differences between the gonadotropin preparations.

2) Aromatase induction

Induction of aromatase activity in Sertoli cells or granulosa cells obtained from immature rats is a good indicator of the *in vitro* biological activity of FSH-containing preparations (26, 27). Puregon induced dose responses parallel to those obtained for the reference preparations IS 70/45 (urine-derived), IS 83/575 (pituitary-derived) and Metrodin (25). The maximal responses obtained with Puregon, Metrodin and the international reference FSH preparations were comparable.

The inhibition of *in vitro* bioactivity by three different monoclonal antibodies directed against either an α- or β-subunit-specific epitope or an α/β-subunit-specific conformational epitope was investigated in a comparative study with Puregon, reference preparation IS 83/575 and Metrodin (25). The amount of each antibody required to neutralise the *in vitro* bioactivity was similar for all three preparations. Although this is not absolute proof of identity, it may be concluded that the FSH proteins in all preparations shared a close structural and functional similarity.

3) Intrinsic LH activity

Most commercially available FSH-containing preparations also contain small or considerable quantities of LH activity due to the presence of hLH, hCG-like material originating from the pituitary, and/or placental hCG (28-30). As a consequence of the biotechnological manufacturing process, Puregon cannot contain LH activity ascribed to the physical presence of any of these hormones.

The capability of Puregon to induce LH-specific biological responses was assayed in the mouse *in vitro* Leydig cell test for the induction of testosterone synthesis. Puregon induced a relatively small increase in testosterone production and at 75 IU/ml, a maximal response of only 30% of that obtained with Metrodin (at 10 IU/ml) was found (25). This effect is most likely induced by a nonspecific interaction of recombinant hFSH with the LH receptor at supraphysiological concentrations. The calculated *in vitro* LH activity of Puregon per IU *in vivo* bioactive FSH was less than 0.025 mIU per IU FSH, which is approximately 100-fold lower than 2.4 mIU per IU FSH of Metrodin. Therefore, the intrinsic LH bioactivity of Puregon must be considered negligible.

4) *In vivo* bioactivity in rats

In the rat ovarian weight gain assay (Steelman-Pohley assay) Puregon induced a dose response parallel to that of a calibrated urinary in-house reference preparation. The specific bioactivity of Puregon (relative to IS 70/45) was approximately 10,000 IU/mg protein, the mass of protein being measured by the Moore and Stein assay (14).

To investigate the effects of Puregon on ovarian activity, the drug was administered to immature hypophysectomized rats. This animal model can be considered analogous to infertile human subjects with low endogenous FSH and LH levels. Subcutaneous administration of Puregon produced a significant dose-dependent increase in ovarian weight and aromatase activity when given for 4 days as a cumulative dose of FSH between 5 and 40 IU (in terms of IS 70/45 *in vivo* bioactivity units). Maximal increases were found with total doses between 20 and 40 IU. Within the dose range applied, Puregon given alone did not affect the plasma estradiol concentration, indicating that circulating androgen levels were too low to act as a substrate for the aromatase enzyme complex.

Bioactivity of Puregon was also assessed by gross histological examination of the ovaries. After treatment with a cumulative dose of 2.5-10 IU Puregon, a dose-dependent increase in the number of large follicles and a gradual shift of small antral to large preovulatory follicles was observed. The latter ovulated after a bolus injection of 10 IU of hCG. In comparison to hypophysectomized vehicle-treated animals, the incidence of atresia diminished, especially in the smallest size class of antral follicles. (25, 31). The number of atretic follicles was reduced with increasing doses of Puregon (31, 32). In the same animal model the supportive effect of LH activity on the FSH bioactivity of Puregon was assessed by the co-administration of increasing quantities of hCG (25, 31, 32). When Puregon, at a total dose of 40 IU, was supplemented with 0.1-10 IU of hCG, a further increase in ovarian weight was observed. This augmentation was significant and hCG dose-dependent. In addition, concomitant administration of hCG had a dramatic, hCG-dependent effect in increasing plasma estradiol. It was shown that minute quantities of LH activity (<2.5 mIU hCG per IU FSH) are sufficient to stimulate the production of significant quantities of androgens, which can subsequently be converted to estrogens.

5) General pharmacodynamics

Possible effects of Puregon on the cardiovascular system, blood gas parameters, hematological parameters, electrocardiogram, and blood sodium and potassium values were assessed after

an intravenous doses of Puregon at 250 IU/kg body weight (approximately 12.5-fold higher than the pharmacologically active daily intramuscular dose) in anesthetized dogs. Possible effects on the cardiovascular system and on regulatory functions of the autonomic nervous system were examined in conscious rabbits. The data indicated that intravenous and intramuscular administration of Puregon to dogs and rabbits at a dose level 250-fold higher than the anticipated maximum daily dose applied in humans, has no significant pharmacodynamic side effects. Administration of Puregon to rabbits and dogs evoked no significant cardiovascular, hemodynamic or behavioral effects.

Animal pharmacokinetics

FSH and LH (like other glycoprotein hormones) occur in a spectrum of isohormones, all with similar immunoreactivity but with varying intrinsic bioactivity and plasma residence time. Gonadotropin concentrations in serum can be assessed either by immunoassay or by *in vitro* bioassay. Whereas an immunoassay only gives information on the mere presence of molecules (quantity), an *in vitro* bioassay gives an indication of the overall intrinsic bioactivity present (quantity and quality). The *in vivo* bioactivity is determined by the intrinsic (*in vitro*) bioactivity and the plasma residence time (reflected by $t_{1/2}$).

Nonclinical pharmacokinetic studies on Puregon were performed in rats and dogs. Animals were dosed with FSH based on the declared *in vivo* bioactivity (in terms of IU) as determined in the rat Steelman-Pohley assay.

Pharmacokinetic parameters of immunoreactive FSH in the rat were very similar for Puregon and Metrodin (Table I). Single-dose intravenous administration (50 IU/kg) revealed no differences in terms of elimination half-life ($t_{1/2elim}$) (approximately 6 h) and AUC (approximately 1000 IU·h/l). No dose-related effects on pharmacokinetics (immunoreactive dose-normalized C_{max} and half-life) were found when comparing intravenous administration of 50 and 250 IU/kg.

In the dog, C_{max} values and terminal $t_{1/2elim}$ (approximately 30 h) for Puregon and Metrodin were also similar after intravenous injection of 25 IU/kg. The much slower elimination rates in dogs compared to rats is most likely related to differences in renal clearance of the compounds. In dogs, the AUC after single-dose intravenous administration for Puregon was significantly lower than for Metrodin. The

Table I: Pharmacokinetics of immunoreactive FSH after administration of Puregon and Metrodin in rat and dog (mean ± SD).

Preparation	Dose* (IU/kg) route	sd/rd	C_{max} (IU/l)	t_{max} (h)	$AUC_{0-\infty}$ (IU·h·l)	$nAUC_{0-\infty}$** (IU·h·l)	$t_{1/2el}$*** (h)	F**** (%)
Rat								
Puregon	50 i.v.	sd	-1	-1	1066 ± 298	126 ± 41	5.7 ± 0.9	-1
Metrodin	50 i.v.	sd	-1	-1	917 ± 238	150 ± 45	6.2 ± 1.0	-1
Humegon	50 i.v.	sd	-1	-1	1084 ± 346	168 ± 53	5.7 ± 0.9	-1
Puregon	250 i.v.	sd	-1	-1	4743 ± 1543	117 ± 51	8.0 ± 1.6	-1
Puregon	50 i.m.	sd	23.4 ± 10.4	7.5 ± 2.6	466 ± 223	54 ± 26	11.4 ± 3.9	42
Metrodin	50 i.m.	sd	28.2 ± 8.7	6.3 ± 2.0	498 ± 142	73 ± 22	10.4 ± 3.8	49
Humegon	50 i.m.	sd	28.8 ± 12.4	6.2 ± 3.1	488 ± 193	78 ± 32	11.8 ± 4.6	46
Puregon	250 i.m.	sd	113.8 ± 46.9	8.3 ± 3.7	2623 ± 1196	68 ± 32	10.7 ± 3.2	58
Puregon	50 i.m.	rd	-2	-2	-2	-2	-2	-2
Metrodin	50 i.m.	rd	26.9 ± 13.3	8.4 ± 3.7	403 ± 176	95 ± 35	10.3 ± 3.4	63
Dog								
Puregon	25 i.v.	sd	283 ± 43	-1	1481 ± 340	7.2 ± 0.4	27.9 ± 3.7	-1
Metrodin	25 i.v.	sd	302 ± 60	-1	2550 ± 500	1.7 ± 1.0	30.4 ± 0.9	-1

*Animals were dosed on the basis of *in vivo* bioactivity; **$nAUC_{0-\infty}$ represent immunoreactive dose-normalized values; ***$t_{1/2}$ values represent terminal elimination half-lives; ****absolute bioavailability calculated from individual real dose AUC values in reference to i.v. administration;sd: single dose; rd: repeated dose; -1: values not calculated; -2: FSH could not be assessed due to antibody formation.

Table II: Pharmacokinetic parameters of immunoreactive FSH in Puregon after single-dose i.v., i.m. or s.c. and repeated-dose i.m. administration to dogs (mean ± SD).

Dose (IU/kg)	sd/rd route	C_{max} (IU/l)	nC_{max}** (IU/l)	t_{max} (h)	$AUC_{0-\infty}$ (IU·h/l)	$nAUC_{0-\infty}$ (IU*h/l)	$t_{1/2el}$*** (h)	CL (l/h/kg)
25/50/75*	rd i.m.	166.5± 14.8	0.3 ± 0.03	2.4 ± 0.05	4255 ± 177	7.5 ± 0.3	37.1 ± 3.8	0.0128
25	sd i.v.	346.3± 99.0	1.83± 0.57	-	1951 ± 504	10.4 ± 3.5	30.8 ± 3.2	0.0085
25	sd i.m.	52.4 ± 0.7	0.3 ± 0.04	6.26± 2.46	1574 ± 53	9.1 ± 1.1	32.9 ± 0.5	0.0094
25	sd s.c.	34.3 ± 13.8	0.16± 0.05	6.69± 1.13	1449 ± 205	7.1 ± 0.7	31.9 ± 0.7	0.0113

*Animals were dosed 25, 50 and 75 IU/kg for three successive periods of 5 days; **nC_{max} and $nAUC_{0-\infty}$ represent immunoreacrive dose-normalized values; ***$t_{1/2}$ values represent terminal elimination half-lives; sd: single dose; rd: repeated dose; - not calculated.

observed difference in clearance rates (0.0122 and 0.0076 l/h/kg for Puregon and Metrodin, respectively) is attributed to the difference in isohormone composition of the two preparations.

After a single intramuscular dose in rats (50 IU/kg) Puregon displayed slow absorption into the circulation. The plasma immunoreactive FSH peaked between 6 and 8 hours after injection. No dose-related effects on absorption were found in rats after injection of 50 IU/kg and 250 IU/kg, as C_{max} values (2.7 *vs.* 2.9 IU/l) normalized for the injected immunoreactive FSH (real) dose and t_{max} values (7.5 *vs.* 8.3 h) were similar (Table I). Similar t_{max} and C_{max} values were also obtained after single-dose intramuscular injection of the reference preparations Metrodin and Humegon (50 IU/kg). Likewise, the extent of absorption was the same. $AUC_{0-\infty}$ values were 498, 488 and 466 IU*h/l for Metrodin, Humegon and Puregon, respectively. Absolute bioavailability after single-dose intramuscular administration ranged from 42-49%. Therefore, the three preparations can be considered to be bioequivalent. Thus, Puregon, Metrodin and Humegon administered to rats as a single intramuscular dose of equal *in vivo* bioactive quantities of FSH display the same pharmacokinetics. This is not surprising since the different preparations have been calibrated in the rat *in vivo* bioassay However, when normalizing the C_{max} and $AUC_{0-\infty}$ values for the immunoreactive dose, significantly lower values for Puregon emerge. This is due to the fact that Puregon is less acidic than Metrodin and thus higher quantities of immunoreactive hormone are needed for Puregon to achieve the same *in vivo* biological activity.

A similar significant difference between the dose-normalized $AUC_{0-\infty}$ of Gonal-F (another recombinant hFSH preparation) and of Puregon has recently been reported (33). Comparison of a single-dose intramuscular and intravenous administration in rats revealed that $t_{1/2elim}$ differed approximately 2-fold, the latter being faster (11 h *vs.* 6 h) (Table I). This was also found for urinary-derived hFSH (Metrodin and Humegon). It is assumed that the terminal declining phase in the plasma-level-*versus*-time curve after intramuscular dosing does not reflect true elimination but rather relatively slow absorption of FSH from the injection site (so-called flip-flop effect). When 50 IU/kg intramuscular Puregon was repeatedly administered for 3 weeks to rats, plasma immunoreactive FSH levels were undetectable after the dosing period (Table I) and no pharmacokinetic parameters could be calculated. The inability to detect recombinant hFSH was probably related to the induction of anti-recombinant hFSH antibodies that most likely interfered with the immunoassay used to quantify FSH. When Metrodin was repeatedly administered intramuscularly (50 IU/kg, once daily for 3 weeks) to rats, plasma FSH levels showed a profile very similar to that obtained after single-dose intramuscular administration, as evidenced by the immunoreactive dose-normalized C_{max} (6.4 *vs.* 4.1 IU/l), t_{max} (8.4 *vs.* 6.3 h) and immunoreactive dose-normalized $AUC_{0-\infty}$ (95 *vs.* 73 IU·h/l) values (Table I).

Plasma levels of Puregon were also determined in dogs after repeated intramuscular dosing of 20 IU/kg for 8 days and after successive intramuscular dosing of 25, 50 and 75 IU/kg each for 5 days (Table II). Steady-state levels of recombinant hFSH were reached within 5 days of treatment. Clearance of FSH after repeated dosing was higher than after a single dose. The immunoreactive AUC after repeated administration (4255 IU·h/l) was lower than the expected value of at least three times the AUC after single-dose administration. Accordingly, the immunoreactive dose-normalized AUC values were slightly different. From these observations it was concluded that repeated dosing does lead to accumulation of FSH, but only of the relatively acidic and not of the relatively basic isohormones. Thus, relatively basic isoforms will not give a major contribution to steady-state hormone levels.

Pharmacokinetics of Puregon in dogs was also studied after subcutaneous, intramuscular and intravenous administration. Absorption via the subcutaneous and intramuscular route were similar in terms of (normalized) C_{max} and t_{max} (Table II). Absolute bioavailability in dogs was not significantly different for the intramuscular and subcutaneous route, being 85% and 74%, respectively. These values were not significantly different from those for the intravenous route. Likewise, clearance of Puregon in dogs was independent of the route of administration as demonstrated by the identical clearance values of approximately 0.01 l/h/kg. Terminal $t_{1/2elim}$ also were not different between the three routes (about 30 h), and were in all cases probably determined by the same proportion of relatively acidic isohormones.

Glycoproteins are thought to be removed from the circulation by binding to one of the following types of receptors, depending on terminal sugar residue of the carbohydrate chains: the asialogalactose receptor in hepatocytes, the SO_4-GalNAc receptor in liver Kupffer and endothelial cells, the ubiquitous mannose phosphate receptor, and the mannose receptor in macrophages and liver endothelial cells (34). The removal of recombinant hFSH was studied after a single dose of intravenously administered Puregon by measuring plasma disappearance of immunoreactive FSH in intact, nephrectomized and ovariectomized rats. The removal of FSH after administration of Metrodin served as a comparison. It was shown that FSH (recombinant or urinary), given intravenously to rats, is distributed into a central volume of distribution of 30-50 ml/kg. Since this value is close to the total plasma volume of the rat and was not affected by surgery, it is concluded that the distribution of the hormone is limited to the plasma water compartment of the animal. After administration of a single intravenous dose to nephrectomized rats a similar, approximately 2- to 3-fold increase in the AUC was observed with both preparations, indicating that the kidney is indeed very important in the clearance of FSH. Removal of the ovaries did not alter pharmacokinetic parameters, indicating the limited role of this organ in the clearance process.

Toxicology

Toxicity testing aims to assess possible hazards and risks of a substance in animals prior to human use. The rat has been used as animal model because this species is known to respond to the pharmacological action of FSH and because of the vast experience obtained with this rodent species in safety studies. In addition, toxicological studies have been carried out in the dog. These models have been proven to be very useful for safety testing and they also respond to the pharmacological action of FSH.

1) Single-dose toxicity

Acute toxicity of Puregon was investigated after intravenous administration of high doses of the drug to rats. Rats were treated with 250 IU/kg or 2500 IU/kg Puregon and monitored during administration and for 2 weeks thereafter. The administered doses are similar to or approximately 10- to 20-fold higher than the pharmacologically active subcutaneous dose in hypophysectomized immature rats (100-200 IU/kg/day), and were approximately 50- and 500-fold in excess of the anticipated maximum daily dose in humans (5 IU/kg). Puregon did not induce any drug-related effects with respect to mortality, clinical signs, vaginal smear cytology, body weight, food and water consumption and clinical laboratory variables, including hematology, blood clotting, blood biochemistry and urinalysis, organ weights, gross anatomy and histopathology. Thus, Puregon at a dose of 500 times the anticipated daily human dose had no acute effects on the rat.

2) Repeated-dose toxicity

Repeated-dose toxicity of Puregon was studied after daily intramuscular administration of the drug to young adult rats and to beagle dogs. In addition to the general safety parameters, toxicokinetic and pharmacodynamic parameters (plasma FSH and estradiol levels, vaginal smear cytology) and the formation of anti-recombinant hFSH antibodies were monitored.

The effect of Puregon was studied after intramuscular administration during 4 consecutive weeks to intact and gonadectomized male and female rats at a dose level of 50 IU/kg/day. This level was slightly lower than the optimal pharmacologically active dose of Puregon in immature hypophysectomized rats (100-200 IU/kg/day) (25), but 10 times higher than the anticipated maximum daily dose in humans of 5 IU/kg. Treatment caused no mortalities. In some females, food and water consumption, and body weight gain and ovarian weight were slightly higher. Plasma levels of estradiol in intact female rats remained normal during treatment. In the majority of rats plasma levels of recombinant hFSH were undetectable 1-2 weeks after daily Puregon administration.

A potential interference of induced antibodies was established in a toxicokinetic study comprising 2-week daily administration of Puregon to male and female rats at dose levels of 25 and 50 IU/kg/day. Increased levels of recombinant hFSH, in accordance with the cumulative dose of Puregon given, were measured up to and including the 10th day of

treatment, the increase being more pronounced in the higher dosage group. With continuing treatment, levels declined in some animals, while at 20 days after the start of the injections, recombinant hFSH levels were undetectable in all animals. The induction of anti-recombinant hFSH antibody levels was monitored by a sensitive immunoassay. Antibody formation was more prominent (earlier onset and higher titres) in females than in males.

In view of this induction of anti-recombinant hFSH antibodies in all rats within 10-14 days post-dosing, it was decided to limit the dosing period in the main toxicity study to 2 weeks. Puregon was administered to intact male and female rats at dose levels of 5, 25, 50 and 500 IU/kg/day. This dose range is similar to or 5- to 100-fold higher than the anticipated maximum daily human dose of 5 IU/kg. No drug-related mortalities were observed and most rats showed a regular estrus cycle pattern. No changes were observed in food consumption, body weight, clinical chemistry and urinalysis parameters. A limited number of observations was considered to be affected in a drug-related manner, *i.e.*, the increase in ovarian weight, the higher number of larger ovaries, a 60% increase in the number of corpora lutea and the 8-fold increase in the number of atretic follicles at the highest dose, and most females in the high-dose group had luteinized follicles. In males the decrease in seminal vesicle weight and the occurrence of spermatogenic granulomas of the epididymal head was apparent. In spite of the many changes caused by the daily administration of Puregon at a dose level of 500 IU/kg, no changes suggestive of toxic properties of the drug were observed. It is concluded that administration of Puregon at dose levels known to exert suboptimal, maximal or supra-pharmacological effects in immature hypophysectomized rats induces no significant toxic effects in rats.

In addition, a 13-week study in female and male beagle dogs was carried out to assess the toxic profile of Puregon after chronic intramuscular administration. At doses of 25 and 50 IU/kg Puregon induced persistent estrus and enlargement of mammae and vulva in all females. In male animals an increased testicular weight was measured at the end of the treatment period. Puregon treatment of females resulted in an increased body weight, higher ovarian and uterine weights, and in anemia and leukocytosis predominantly halfway and at the end of the treatment period. Transient thrombocytopenia and increased cholesterol values were also noted in female animals. Puregon exerted no effects on food consumption, ophthalmological parameters and urinalysis. Most drug-treated animals showed lower adrenal weights. Gross anatomy and histopathology of the female genital organs and mammary glands revealed changes consistent with ovarian stimulation. However, the process of ovulation seemed to fail. In most animals Puregon treatment resulted in the induction of anti-recombinant hFSH antibodies. Although a dose relationship was found, there was considerable variation in terms of the extent, time course and persistence of the immunoresponse. Thus, no toxic effects were observed in rats and dogs with doses up to 100-fold in excess of the anticipated maximum daily human dose.

3) Local tolerance

Rats treated intramuscularly with 50 IU/kg or intravenously with 250 IU/kg showed no drug-related abnormalities in clinical signs, body weight, gross anatomy or in histopathological parameters at 5, 24 and 72 hours after drug administration. The few changes observed at the site of injection were minor lesions and are considered to be traumatic in origin. Puregon is, therefore, well tolerated when given to rats in a single dose that is ≥ 10-fold higher than the maximum pharmacologically active dose in rats and 50-fold higher than the maximum human daily dose (5 IU/kg). Absence of local reactions was also shown in the repeated-dose studies in rats and dogs receiving daily intramuscular injections of Puregon.

4) Oncogenic and carcinogenic potential

The potential presence of oncogenic viruses or oncogenic DNA was investigated at different stages of the manufacturing process of the CHO cell line used for the production of Puregon. In addition, the removal of adventitious viruses and DNA at different stages of the purification of Puregon has been investigated by performing spiking experiments. The outcome in terms of clearance factors obtained for viruses and DNA and in terms of the absolute DNA level in the pharmaceutical product provides a sufficient guarantee for the safety of Puregon for human use. Puregon, like existing commercial Metrodin preparations, will be given to female subjects for only limited time periods. Therefore, lifetime studies to investigate the carcinogenic potential of Puregon are not considered to be useful.

5) Mutagenic potential

The active substance of Puregon is a glycoprotein lacking structural alerts associated with mutagenic activity. In addition, the reported small differences in carbohydrate chain structure between recombinant hFSH and natural hFSH are considered irrelevant with respect to its mutagenic potential. In view of the anticipated maximum daily human dose and the extreme purity of Puregon, less than a total amount of 0.3 µg of non-FSH related impurities will be administered. This very small quantity cannot

constitute a genotoxic risk, in particular when the level of individual contaminating proteins is expected to be even in the low nanogram or picogram range. Although there is no scientific basis for any suspicion of mutagenicity of recombinant hFSH or production process-related contaminants, the following tests were carried out.

In an Ames test with four strains of *Salmonella typhimurium* (TA98, TA100, TA1535 and TA1537) and one strain of *Escherichia coli* (WP2 uvrA pKM101) with Puregon in quantities ranging from 8-5000 μg per plate (corresponding to 80-50,000 IU of FSH), recombinant hFSH neither exerted cytotoxic effects nor induced an increase in the number of revertants in comparison with vehicle-treated bacteria. In a chromosome aberration test in peripheral human lymphocytes *in vitro*, cells were incubated with Puregon at concentrations of 0.01, 0.1 and 1 IU/ml, dose levels corresponding to respectively 1, 10 and 100 times the maximum human plasma level. On the basis of the relative mitotic inhibition found, Puregon did not cause marked cytotoxicity. Puregon showed no significant increase in the frequency of cells with chromosomal aberrations as was found with the positive control compounds mitomycin C and cyclophosphamide. Thus, Puregon displays no mutagenic potential.

6) Immunogenic potential

An important safety aspect of biotechnologically prepared proteins is the potential immunogenicity of the active substance or potential proteinaceous contaminants, notably in the case of Puregon culture medium- or CHO cell-derived protein contaminants (low nanogram or picogram range). When human proteins such as FSH are administered to animals, it is not surprising that such non-self proteins induce antibody formation. Factors that influence the degree of antibody formation are the animal species used, the dose and frequency of the foreign protein given, the conditions under which the protein is given (immunochallenging or not) and the conformational state of the protein (aggregated or not). In the rat, no anti-recombinant hFSH antibody formation was observed after a single intravenous administration of Puregon. Via the intramuscular route, Puregon induced anti-recombinant hFSH antibodies in a single animal within a period of 4 days and after 7 days in the majority of the rats dosed at 500 IU/kg. Beyond 2 weeks all animals dosed at 50 or 500 IU/kg were positive for the presence of anti-recombinant hFSH antibodies. The antibody response in female rats occurred at earlier stages and showed higher titers than that in male rats. As a result of the presence of circulating antibodies, plasma recombinant hFSH levels were undetectable within 10-20 days after treatment, dependent on the dosage applied. In the 13-week toxicity study in beagle dogs, most animals treated with Puregon showed the induction of anti-recombinant hFSH antibodies. Although a dose relationship was found, there was considerable variation in terms of the extent, time course and persistence of the immunoresponse.

Human Pharmacokinetics

Single-dose pharmacokinetics

In a crossover design, gonadotropin-deficient, but otherwise healthy subjects were treated intramuscularly with 300 IU Puregon and 300 IU Metrodin (35). C_{max}, t_{max}, $t_{1/2elim}$ and $AUC_{0-\infty}$ were calculated for each treatment on the basis of immunoreactive FSH plasma levels (Table III).

Serum FSH concentrations in the range of C_{max} were reached within 12 hours after administration, remaining at that level for 24-48 hours. There was a statistically significant difference in the rate of absorption between male and female subjects: in males C_{max} was reached in about half the time needed to reach C_{max} in women, and the mean C_{max} was about 70% higher than in women. No significant sex differences could be established with respect to $t_{1/2}$ and $AUC_{0-\infty}$. Gender differences in drug absorption after administration of aqueous solutions, specifically into the gluteus maximus, have been described. They are thought to be related to gluteal fat thickness in women, which exceeds that in men (36). The extent of absorption of immunoreactive FSH was significantly higher for Metrodin than for Puregon (C_{max} and $AUC_{0-\infty}$ both about 65% of those after Metrodin). The differences with respect to t_{max} and $t_{1/2}$ were not statistically significant.

Multiple-dose pharmacokinetics

The pharmacokinetics of Puregon after repeated intramuscular administration was investigated in open, group-comparative studies. In the first study, Puregon was administered in weekly rising doses of 75, 150 and 225 IU in gonadotropin-deficient but otherwise healthy volunteers. From the individual data it appears that relatively high concentrations of FSH were reached within about 12 hours after administration. Serum FSH increased dose proportionally in the course of treatment and steady state for each dose level was reached after 3-5 daily doses. As expected, serum LH concentrations did not increase during the study, confirming that Puregon lacks intrinsic LH activity. Due to the relatively long $t_{1/2elim}$, serum concentrations of FSH at steady

Table III: FSH pharmacokinetics after single intramuscular dosing of 300 IU Puregon and Metrodin (mean ± SD).

Parameter	Male		Female		Probability from ANOVA	
	Puregon (n = 6)	Metrodin (n = 4)	Puregon (n = 8)	Metrodin (n = 5)	Male *vs.* Female	Puregon *vs.* Metrodin
C_{max} (IU/l)	7.4 ± 2.8	11.6 ± 1.7	4.3 ± 1.7	7.2 ± 2.3	0.0072	0.0002
t_{max} (h)	14.3 ± 7.5	9.2 ± 2.1	26.9 ± 5.4	21.1 ± 10.9	0.0004	0.3778
$t_{1/2}$ (h)	32.1 ± 11.6	43.9 ± 9.9	43.9 ± 14.1	37.2 ± 9.3	0.2685	0.2785
$AUC_{0-\infty}$ (IU·h/l)	452 ± 183	764 ± 190	339 ± 105	547 ± 127	0.0582	0.0001

Table IV: FSH pharmacokinetics after repeated intramuscular dosing of Puregon and Metrodin (mean ± SD).

Parameter	Puregon 75 IU (n = 9)	Puregon 150 IU (n = 7)	Puregon 225 IU (n = 8)	Metrodin 150 IU (n = 8)
C_{max} (IU/l)†	4.7 ± 1.5	9.5 ± 2.6	11.3 ± 1.8	10.7 ± 2.2
t_{max} (h)†	8.2 ± 4.3	10.9 ± 6.3	11.3 ± 8.0	4.8 ± 3.0
AUD_{0-24} (IU·h/l)‡	97 ± 22	204 ± 49	242 ± 44	231 ± 44
$t_{1/2}$ (h)†	26.9 ± 7.8	30.1 ± 6.2	28.9 ± 6.5	30.3 ± 4.1
Clearance (l/h/kg)	0.011 ± 0.002	0.010 ± 0.002	0.013 ± 0.002	0.010 ± 0.001

†Measured after the last injection (for $t_{1/2}$ during the linear phase of the curve); ‡determined after the last injection by using the trapezoidal rule.

state were higher than after single administration. Based on C_{min} (FSH concentration just prior to each dosing), an accumulation factor of approximately 1.5-2.5 could be estimated. In gonadotropin-deficient subjects, significantly different disappearance half-lives were found between female and male volunteers ($t_{1/2elim}$ 39 ± 8 and 48 ± 5 h, respectively) (37, 38).

The second multiple-dose pharmacokinetic study was performed in 4 groups of 9 healthy female volunteers whose endogenous gonadotropin production was suppressed by a high-dose oral contraceptive pill (Lyndiol), and who received 75, 150 or 225 IU Puregon or 150 IU Metrodin (Table IV).

Statistical analysis based on ANOVA indicated dose proportionality for the 75 IU and 150 IU Puregon treatments. Based on C_{max} and AUD (AUC of one dosing interval), the relative availability after dosing of 225 IU Puregon seemed to be lower, although the mean (dose-normalized) C_{min} levels were reported to be dose-proportional. In contrast to the data obtained after single administration, the bioavailability of immunoreactive FSH (reflected by AUD and C_{max}) after repeated administration of 150 IU Puregon and Metrodin were in the same range, although bioequivalence could not be proven due to a high variability. With respect to $t_{1/2elim}$ and clearance, Puregon and Metrodin proved to be bioequivalent.

Bioavailability

To assess the absolute bioavailability of Puregon in healthy female volunteers of reproductive age, pituitary suppression is required to prevent interference of endogenous FSH with the determination of exogenous FSH. Pituitary suppression was obtained by using a high-dose oral contraceptive pill (Lyndiol). In an open, randomized, three-way crossover study the absolute bioavailability was assessed after single-dose intramuscular (buttock) and subcutaneous (abdominal wall) administration of 300 IU Puregon to healthy female subjects. Based on serum immunoreactive FSH, C_{max}, t_{max}, $AUC_{0-\infty}$ and the absolute bioavailability (F) were calculated (Table V).

With respect to the extent of absorption, measured by the AUC of immunoreactive FSH, intramuscular and subcutaneous administration proved to be bioequivalent. For both routes the absolute bioavailability was found to be about 77%. Although the means were almost identical, no bioequivalence could be proven with respect to C_{max} and t_{max}. For the latter, this may have been caused by the high intrasubject variability. The intersubject variability

Table V: FSH pharmacokinetics after a single i.v., i.m. or s.c. dose of 300 IU Puregon (mean ± SD).

Parameter	Intravenous (n = 5)	Intramuscular (n = 5)	Subcutaneous (n = 5)	Bioequivalence i.m. *vs.* s.c.
C_{max} (U/l)	42.99 ± 3.89	6.86 ± 0.90	5.41 ± 0.72	not proven
t_{max} (h)	n.a.	18.15 ± 10.15	17.38 ± 7.68	not proven
$AUC_{0-\infty}$ (IU·h/l)	587.8 ± 202.7	445.7 ± 135.7	455.6 ± 141.4	proven
F (%) (95% CI)	n.a.	76.4 [69.7-83.8]	77.8 [70.9-85.2]	-

n.a. = not applicable.

Table VI: Disposition of subjects participating in the pilot efficacy study (43).

	Treatment group				
Variable	I (n = 9)	II (n = 9)	III (n = 11)	IV (n = 11)	V (n = 10)
GnRH agonist	none	Buserelin	Buserelin	Triptorelin	Triptorelin
Protocol	-	short	long	long	long
Dose		4 x 150 µg/d	4 x 150 µg/d	3.75 mg	100 µg
Route		intranasal	intranasal	intramuscular	subcutaneous
Start Puregon (day)	3	3	>15	>15	>15

in C_{max} after intramuscular administration was higher than after subcutaneous administration (43% *vs.* 13%). This may be attributable to the high variation between subjects with respect to the presence of gluteal fat.

Although bioequivalence could only be proven with respect to the extent of absorption, these data indicate that single-dose intramuscular and subcutaneous administration lead to highly similar immunoreactive FSH serum concentration curves.

Clinical Studies

In 1992, the first pregnancy and birth of a healthy baby after treatment with Puregon in controlled ovarian hyperstimulation with IVF/ET (39, 40) and ovulation induction (41, 42) were achieved. Clinical studies performed since then have confirmed that Puregon is a safe and efficacious drug.

Controlled ovarian hyperstimulation

A pilot efficacy study was carried out to evaluate whether Puregon may be combined with various treatment regimens of GnRH agonists, inducing various degrees of pituitary suppression (43). Eligible subjects were allocated to 5 different treatment groups. GnRH agonist treatment with buserelin (Suprecur, Hoechst) in both a long and short GnRHa protocol, or triptorelin (Decapeptyl, Ferring) in a long protocol i.m. or s.c., was started at day 1 of the menstrual cycle. During the first treatment days the Puregon dose was fixed to 75 IU in Group II, and to 150-225 IU in Groups I, III, IV and V (Table VI).

In total, 51 healthy females of infertile couples started one treatment cycle with Puregon. One subject was cancelled because of a premature LH surge. The pharmacodynamics and clinical outcome could be assessed in 50 subjects.

At the start of Puregon treatment the concentrations of endogenous FSH and LH were lower in subjects treated with buserelin or triptorelin in a long protocol (groups III, IV and V) than in groups I and II (Table VII). In the former groups, endogenous LH concentrations were further decreased at the day of hCG administration, suggesting a progressive pituitary suppression during treatment.

Regardless of the concentration of serum LH, Puregon treatment resulted in normal rises of estradiol in all groups. At the day of hCG administration serum concentrations of estradiol were within the normal preovulatory range and correlated well with the degree of multiple follicular development. The median number of oocytes recovered per group varied from 7-11 and all but 2 cumulus-corona complexes were classified as mature. The median fertilization rate between the groups ranged from 40-73% and the median cleavage rate from 73-100%. In total, 43 women had an embryo transfer; clinical pregnancies were established in 10 women, 8 of whom had an ongoing pregnancy (19%).

Table VII: Mean results of pharmacodynamic parameters in the pilot efficacy study (43).

Parameter	Treatment group				
	I (n = 9)	II (n = 9)	III (n = 11)	IV (n = 11)	V (n = 10)
	Serum concentrations before start of Puregon treatment [median (range)]				
FSH (IU/l)	9 (6-14)	6 (3-9)*	4 (1.7-10)	3 (1-11)	4.4 (<0.9-7)
LH (IU/l)	6.7 (5.6-12–0)	4.9 (2.4-7.3)	3.2 (0.1-4.6)	2.8 (2.3-4.9)	2.4 (1.5-4.5)
E2 (ng/l)	39 (17-59)	33 (21-38)	33 (14.2-65)	27 (<15-34)	24 (<20-266)
	Serum concentrations at the day of hCG administration [median (range)]				
FSH (IU/l)	21 (14-26)	13 (4-17)	15 (4-24)	17 (9.1-27)	17 (8-30)
LH (IU/l)	5.1 (1.2-20.0)	2.3 (<0.5-12.0)	1.3 (<0.5-7.1)	1.2 (0.8-3.5)	1.6 (<0.5-2.7)
E2 (ng/l)	1101 (684-2467)	1899 (948-2640)	1773 (711-2958)	1768 (781-2952)	1531 (893-3350)

*Measured at the start of GnRH agonist treatment.

The results from this study indicated that Puregon, alone or in combination with different GnRH agonist regimens, is effective in COH. Stimulation leads to adequate preovulatory estradiol concentrations, irrespective of the degree of pituitary (LH) suppression.

A pivotal IVF study (European Puregon Collaborative IVF Study) was set up to compare the efficacy and safety of Puregon and Metrodin in infertile pituitary-suppressed women undergoing COH followed by IVF/ET (44). The study was designed as a randomized (Puregon:Metrodin = 3:2), assessor-blind, group-comparative, multicenter study with over 1000 subjects. Both groups received buserelin (Suprecur, Hoechst; 4x150 µg/day intranasally), starting on day 1 of the cycle. Treatment with Puregon or Metrodin started only after confirmation of down-regulation of the pituitary. From treatment days 1-4, subjects received an i.m. dose of 150 or 225 IU Puregon or Metrodin, once daily. Thereafter, the dose and treatment duration were individualized, guided by ovarian response. Stimulation was continued until there was ultrasonic evidence of adequate multiple follicular development, *i.e.*, at least 3 follicles of ≥17 mm. When this criterion was met, hCG was administered and follicle puncture and oocyte pick-up were carried out.

In each of the 18 centers, one of the main efficacy parameters , *i.e.*, the mean number of oocytes per aspiration, was higher in the Puregon group (Fig. 1). This resulted in a significantly greater overall mean number of oocytes per aspiration (10.8 *vs.* 9.0,

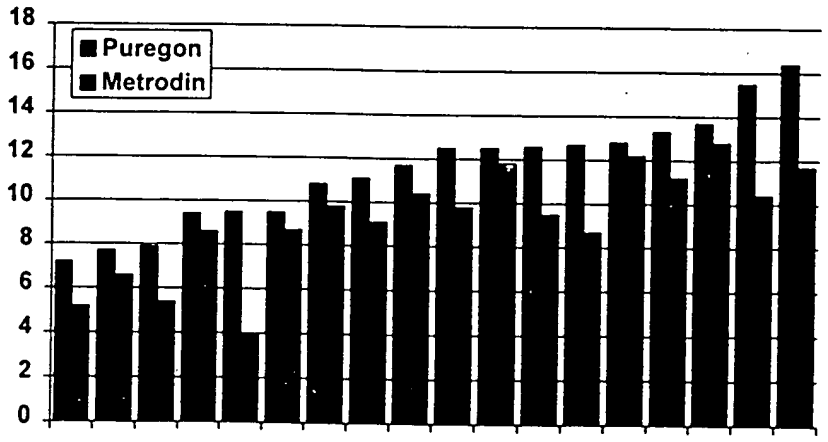

Fig. 1. Mean number of oocytes retrieved with Puregon and Metrodin in 18 study centers.

Table VIII: Results of Puregon vs. Metrodin in IVF/ET using buserelin intranasally as GnRH agonist (44).

Parameter	Puregon (n = 585)	Metrodin (n = 396)	*p*-value
Efficiency			
Total FSH dose (IU)	2138	2385	$p < 0.0001$
Duration of treatment (days)	10.7	11.3	$p < 0.0001$
Efficacy			
Maximum serum estradiol (pmol/l)	6084	5179	$p < 0.0001$
Total number of oocytes retrieved	10.8	9.0	$p < 0.0001$
Number of mature oocytes retrieved	8.6	6.8	$p < 0.0001$
Number of follicles $\geq$ 15 mm	7.5	6.7	$p = 0.0002$
Number of follicles $\geq$ 17 mm	4.6	4.4	$p = 0.09$
Number of high-quality embryos	3.1	2.6	$p = 0.003$
Ongoing pregnancy rate per cycle	22.2%	18.2%	$p = 0.13$
Ongoing pregnancy rate per cycle including frozen embryo cycles	25.7%	20.4%	$p = 0.05$

p <0.0001; Table VIII). This is reflected in significantly greater mean numbers of mature oocytes and high quality (type 1 and type 2) embryos found in the Puregon group ($p = 0.003$). Although the number of embryos transferred was standardized in both groups (*i.e.*, a maximum of 3 embryos were transferred), a slight (4%), although not statistically significant, difference in ongoing pregnancy rate was found in favor of the Puregon group (Table VIII).

For the subjects with oocyte retrieval, the efficiency parameters, *viz.* the total FSH dose and the mean number of treatment days necessary to reach the criteria for hCG administration, were statistically significantly lower (p <0.0001) in the Puregon group compared to Metrodin (2138 *vs.* 2385 IU and 10.7 *vs.* 11.3 days, respectively). In addition to the fact that Puregon treatment required a lower total dose of FSH and a shorter treatment period, a number of other parameters differed in favor of Puregon. For follicles >15 mm there was a statistically significant difference (7.5 *vs.* 6.7 for Puregon and Metrodin, respectively, $p = 0.0002$). The mean maximal serum estradiol concentration was also significantly higher in the Puregon group (6084 *vs.* 5179 pmol/l, p <0.0001). In 11 out of the 18 centers immunoreactive FSH in serum was measured. The mean immunoreactive FSH concentration just before, or on the day of hCG administration was significantly higher in the Metrodin group than in the Puregon group (12.1 *vs.* 11.5 IU/l, $p = 0.03$).

When the results of the frozen embryo cycles are included (143 cycles with Puregon and 88 with Metrodin), the cumulative ongoing pregnancy rate per cycle was 25.7% with Puregon and 20.4% with Metrodin. Statistical analysis of the difference in cumulative pregnancy rate after the FSH treatment cycle, including frozen embryo cycles, showed that the difference of 5.3% in favor of the Puregon-treated women was statistically significant ($p = 0.05$).

A total of 19.3% of subjects in the Puregon group and 15.6% of subjects in the Metrodin group experienced at least one adverse experience (AE), of whom with Puregon 9.3% were considered drug-related as compared to 6.8% in the Metrodin group. In both treatment groups the majority of AEs were classified as "reproductive disorders" (10 7% *vs.* 9.5%). Ovarian hyperstimulation syndrome leading to hospitalization occurred in 3.2% with Puregon and in 2.9% with Metrodin. Statistical analysis of the total incidence of OHSS (5.2% *vs.* 4.3%) revealed no statistically significant difference between the Puregon and the Metrodin groups. Neither anti-FSH antibodies nor anti-CHO cell-derived protein antibodies were found in any of the subjects for whom serum was available, even after 3 treatment cycles.

From this study it was concluded that Puregon was more efficacious than Metrodin in controlled ovarian hyperstimulation in infertile pituitary-suppressed women who were undergoing IVF/ET, as assessed by the number and the degree of maturity of oocytes retrieved and the number of type 1 and 2 embryos after the first treatment cycle. Furthermore, Puregon was more efficient because oocyte retrieval was achieved using a lower total dose in a shorter treatment period. The pregnancy rate (including frozen embryo cycles) was significantly higher in the

Table IX: Results of Puregon vs. Metrodin in IVF/ET using triptorelin subcutaneously as GnRH agonist (45).

Parameter	Puregon (n = 57)	Metrodin (n = 33)	*p*-value
Efficiency			
Total FSH dose (IU)	2265	2213	n.s.
Duration of treatment (days)	10.2	10.3	n.s.
Efficacy			
Maximum serum estradiol (pmol)	7551	5514	n.s.
Total number of oocytes retrieved	9.7	8.9	n.s.
Number of mature oocytes retrieved	8.1	6.9	n.s.
Number of follicles $\geq$ 15 mm	7.3	7.2	n.s.
Number of follicles $\geq$ 17 mm	5.4	5.5	n.s.
Number of high-quality embryos	3.7	4.0	n.s.
Ongoing pregnancy rate per transfer	34.0%	18.8%	n.s.
Ongoing pregnancy rate cycle	30.2%	17.4%	n.s.

n.s.: not significant

Puregon group compared to Metrodin. There was no difference between Puregon and Metrodin with respect to safety.

An additional study was performed using another GnRH agonist. The experimental design of this study was analogous to the design of the pivotal IVF study (44), except for the fact that triptorelin instead of buserelin was used for pituitary suppression (45). Treatment with Puregon or Metrodin started only after confirmation of down-regulation of the pituitary, and from treatment days 1-4, subjects received a daily i.m. dose of 150 or 225 IU Puregon or Metrodin. Thereafter, the dose and duration were individualized, guided by ovarian response.

In total, 60 subjects were randomized to Puregon and 39 to Metrodin (randomization 3:2). The two treatment groups were similar with respect to infertility, demographic and menstrual cycle characteristics. The main cause of infertility was of tubal origin. The main efficacy parameter, *i.e.*, the total number of oocytes, was comparable for both groups. After standardization of the total number of embryos transferred (2.9 for the Puregon group and 2.5 for the Metrodin group), higher pregnancy rates (although not statistically significant) were seen in the Puregon group; the ongoing pregnancy rates per attempt and per transfer were 30.2% *vs.* 17.4% and 34.0% *vs.* 18.8% for the Puregon and Metrodin groups, respectively. The efficiency parameters, *viz.* total FSH dose and the duration of treatment, were not different in the Puregon and Metrodin treatment groups (Table IX).

A total of 15.8% of subjects experienced at least one AE (including subjects with SAEs) in the Puregon group as compared to 12.1% in the Metrodin group. 12.3% of subjects in the Puregon group and 6.1% in the Metrodin group had drug-related AEs. AEs leading to hospitalization were reported in 3 subjects in the Puregon group (5.3%, all with OHSS) and in 1 subject in the Metrodin group (3.0%; abdominal pain). Neither anti-FSH antibodies nor anti-CHO cell-derived protein antibodies were found in any of the subjects for whom serum was available. It was concluded that there was no significant difference in efficacy and safety between Puregon and Metrodin treatment in controlled ovarian hyperstimulation in infertile triptorelin pituitary-suppressed women undergoing IVF/ET.

In order to investigate local safety aspects associated with subcutaneous administration of Puregon, one pivotal study was performed (46). This phase III study was primarily designed to assess the local tolerance and safety, and secondarily, to compare the efficacy of Puregon administered either intramuscularly or subcutaneously in infertile women undergoing IVF. The study was designed as a randomized (2:3 ratio between the i.m. and s.c. group), open, group-comparative, multicenter study. Local tolerance parameters (bruising, redness, itching, pain and swelling) were scored by the subject as either none, mild, moderate or severe. Both groups received buserelin (Suprecur, Hoechst; 4x150 µg/day intranasally) for pituitary down-regulation, starting on day 1 of the cycle. Treatment with Puregon started after confirmation of down-regulation. From treatment days 1-4, subjects received daily 150 or 225 IU Puregon i.m. or s.c. Thereafter, the dose and duration were individualized, as guided by

Table X: Results of Puregon i.m. vs. s.c. administration in IVF/ET using buserelin intranasally as GnRH agonist (46).

Parameter	Intramuscular (n = 7)	Subcutaneous (n = 118)	*p*-value
Efficiency			
Total no. of ampules/vials used	29.8	28.2	n.s.
Duration of treatment (days)	9.9	9.7	n.s.
Efficacy			
Mean serum estradiol on day hCG (pmol/l)	5509	7109	n.d.
Total number of oocytes retrieved	9.8	10.4	n.s.
Number of mature oocytes retrieved	8.2	8.6	n.s.
Number of follicles ≥ 15 mm	8.1	8.5	n.s.
Number of follicles ≥ 17 mm	4.3	5.0	n.s.
Number of high-quality embryos	4.3	3.8	n.s.
Ongoing pregnancy rate per transfer	30.1%	29.3%	n.s.
Ongoing pregnancy rate per cycle	27.1%	26.1%	n.s.

n.s.: not significant; n.d.: not determined

ovarian response. The incidences after i.m. injection of overall local symptoms, bruising, pain, redness, swelling and itching were 63.6, 37.7, 31.2, 13.0, 7.8 and 6.5%; after s.c. injection the corresponding figures were 68.6, 54.2, 28.0, 16.1, 5.9 and 3.4%. Only bruising was significantly lower in the i.m. group (95% confidence interval -29.7% to -2.6%; p = 0.019), most likely because after s.c. administration bruising is more easily visible due to the more superficial injection site. The results regarding the efficacy after i.m. or s.c. Puregon treatment were similar (Table X), and also very similar to the results reported by others (44, 45). This is in accordance with the expectations based on the bioequivalence reported between the two routes.

In total, 6.5% of subjects in the i.m. group and 12.7% in the s.c. group experienced AEs. A total of 3.6% of subjects had drug-related AEs: 2 subjects in the Puregon i.m. group (2.6%) and 5 subjects in the Puregon s.c. group (4.2%). One subject treated in the i.m. (1.3%) and 6 subjects in the s.c. group (5.1%) experienced SAEs. Most frequently reported AEs were OHSS (i.m., 2.6%; s.c., 5.9%) and ectopic pregnancy (i.m., 1.3%; s.c., 1.7%). Serum anti-FSH antibodies were not found in any of the subjects for whom serum was available.

There were no statistically significant differences in the occurrence of most local tolerance symptoms (pain, redness, swelling, and itching) and safety parameters between i.m. and s.c. administration of Puregon in infertile women. Overall local tolerance, however, was not statistically significantly different. No clinically relevant differences in efficacy between i.m. and s.c. administered Puregon were found.

Ovulation induction

In order to support the claim for this indication, a pivotal ovulation induction study was performed (47). This phase III study was designed to compare the efficacy and safety of Puregon to Metrodin for induction of ovulation in patients with chronic anovulation (WHO group II) who failed to ovulate and/or conceive during clomiphene citrate treatment. The study was designed as a randomized (Puregon:Metrodin = 3:2), assessor-blind, group-comparative, multicenter study. The study period covered a maximum of 3 treatment cycles. When significant follicular development was observed during the course of treatment, the daily FSH dose was not increased. This dose was maintained until a follicle diameter ≥ 18 mm was observed or 2 or 3 follicles had reached a diameter of ≥ 15 mm. Ovulation was then induced by administration of 10,000 IU of hCG. The two treatment groups were similar with respect to demographic and menstrual cycle characteristics. The main preovulatory criteria were 2 or 3 follicles ≥ 15 mm or a dominant follicle ≥ 18 mm.

With Puregon, a statistically significantly greater number of follicles ≥ 12 mm was found in the first treatment cycle and mean preovulatory estradiol

Table XI: Results of Puregon vs. Metrodin in ovulation induction (WHO-group II) (47).

Parameter	Puregon (n = 105)	Metrodin (n = 67)	*p*-value
Efficiency			
Median total dose (IU)[1]	750	1035	$p < 0.001$
Median duration of treatment (days)[1]	10.0	13.0	$p < 0.001$
Efficacy			
Med. maximum estradiol (pmol/l)[1,2]	1400	1000	n.s.
Number of follicles $\geq$ 12 mm[1,2]	3.6	2.6	$p < 0.001$
Number of follicles $\geq$ 15 mm[1,2]	2.0	1.7	n.s.
Cancellations	11.4%	10.4%	n.s.
Cumulative ovulation rate	95%	96%	n.s.
Cumulative pregnancy rate	27%	24%	n.s.

[1]In the first cycle; [2]on day of hCG administration.

levels were higher. These results were obtained using significantly fewer ampoules in a significantly shorter treatment period. Statistical significance was proven for the efficiency parameters in the first treatment cycle. No statistically significant differences were found regarding the distribution of first time ovulations, even in the most conservative statistical approach, in which dropouts are considered as subjects who did not achieve ovulation in the subsequent cycles. Cumulative ovulation rates after 3 cycles were also not different for Puregon and Metrodin (Table XI).

AEs were reported in 29.5% of subjects in the Puregon group and in 34.3% in the Metrodin group. For 7 subjects on Puregon and 5 on Metrodin the AEs were serious. There were 8 subjects on Puregon (7.6%) and 3 on Metrodin (4.5%) with OHSS and one of these in the Puregon group was reported as an SAE. The difference was not statistically significant. In each treatment group, about 18% of AEs were considered as drug-related. Serum anti-FSH antibodies were not found in any of the subjects for whom serum was available, even after 3 treatment cycles.

Based on the data obtained, it is concluded that there are no significant differences in the efficacy and safety of Puregon and Metrodin for induction of ovulation in subjects with chronic anovulation (WHO group II) who failed to ovulate and/or conceive during clomiphene citrate treatment. With Puregon, however, ovulation was obtained with significantly fewer ampoules in a significantly shorter treatment period, indicating a higher efficiency in ovulation induction. The higher efficiency of Puregon, and the fact that a greater number of follicles $\geq$ 12 mm were stimulated, suggest that Puregon treatment may be started at a lower dose than Metrodin and maintained at this lower dose for a longer period of time.

Male hypogonadotropic hypogonadism

Fertility in men depends on both LH and FSH to induce androgen production and spermatogenesis. In hypogonadotropic hypogonadal men with GnRH deficiency or hypopituitarism, administration of exogenous urinary gonadotropins stimulates both androgen and sperm production (48). A pharmacokinetic study indicated that serum FSH increases in a dose-proportional fashion after intramuscular administration in hypogonadotropic hypogonadal men (35). Recently, the first case of successful induction of spermatogenesis with Puregon in a postpubertal hypogonadotropic hypogonadal male was reported (49). After 18 weeks of treatment, the first sperm was observed in the ejaculate of this patient, reaching normal levels in the further course of treatment. Puregon was well tolerated and without side effects.

Conclusions

In controlled ovarian hyperstimulation programs followed by IVF/ET, Puregon proved to be more efficacious than Metrodin. Puregon treatment resulted in a significantly greater number of oocytes. The ongoing pregnancy rate of Puregon was significantly higher than that of Metrodin in the European Puregon Collaborative IVF Study after the inclusion of frozen embryo cycles. Furthermore, these results were achieved with a lower total dose within a shorter treatment period. This indicates a higher efficiency of Puregon in IVF.

In ovulation induction, ongoing pregnancy rates (per attempt and per transfer) were not statistically different for the Puregon and Metrodin groups. Puregon treatment, however, required a 30% lower total FSH dose and a shorter treatment period to achieve the same results. This indicates a higher efficiency of Puregon also in ovulation induction.

S.c. and i.m. administration of Puregon were equally efficacious, as was expected because there are no significant pharmacokinetic differences regarding bioavailability and extent of absorption. Puregon is efficacious when used without and with different GnRH agonists.

I.m. and s.c. administrations of Puregon were well tolerated, and the safety pattern is similar to that of Metrodin i.m. With respect to the most common adverse experience, OHSS, statistically significant differences were not reached in any of the studies. Specific antibody formation against either FSH or CHO cell-derived proteins was not found in any of the Puregon-treated subjects analyzed, even after 3 treatment cycles.

These results indicate that this biotechnologically manufactured gonadotropin preparation is safe and effective in controlled ovarian hyperstimulation and ovulation induction, using both the i.m. and s.c. routes of administration.

Acknowledgements

The following investigators contributed to the clinical program of Puregon: Belgium: J. Donnez, Cliniques Universitaires St. Luc, Service de Gynecologie, Brussels; P. Devroey, A. van Steirteghem, J. Smitz, Center for Reproductive Medicine, Academic Hospital Free University, Brussels. Denmark: P. Hornnes, Rikshospitalet, Department of Gynaecology, Fertility Clinic, Copenhagen. Finland: A. Tiitinen, N. Simberg, M. Tulppala, M.T. Seppälä, Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, Helsinki. France: B. Camier, Centre de Procréation Médicalement Assistée, 80054 Amiens; J. Cohen, J. Belaïsch-Allart, L. Nicolle, Centre Hospitalier Intercommunal Jean Rostand, 923311 Sévres; B. Hedon, C. Humeau, L. Lafont, Centre Hospitalier et Universitaire de Montpellier, 34295 Montpellier; J.N. Hugues, I. Cedrin Durnerin, Hôpital Jean Verdier, 93143 Bondy; P. Lopes, B. Charbonnel, P. Barriere, F.X. Laurent, Hotel Dieu, 40000 Nantes; J.R. Zorn, I. Matheron, Clinique Universitaire Baudelocque, 75014 Paris. Germany: K. Diedrich, C. Diedrich, Universitäts-Frauenklinik Bonn; L. Wildt, E. Siebzehnrübl, B. Munzer, Universitäts-Frauenklinik Erlangen; O. Naether, Lornsenstrasse 4, Postfach 500906, Hamburg. Greece: B.C. Tarlatzis, J. Bontis, S. Lagos, H. Bili, D. Bakratsa, S. Mantalenakis, Infertility and IVF Centre "Geniki Cliniki", Thessaloniki. Ireland: R.F. Harrison, U. Kondaveeti, B. Hennelly, A. Gordon, L. Drudy, E. Cottell, A. MacMahon, Human Assisted Reproduction Ireland, RCSI Academic Department of Obstetrics and Gynaecology, Rotunda Hospital, Dublin. Israel: Z. Shoham, A. Barash, P. Golan, I. Segal, L. Sindel, V. Insler, Department of Obstetrics and Gynaecology, Kaplan Hospital, Rehovoth. The Netherlands: T. van Dessel, P. Donderwinkel, B. Fauser, Department of Obstetrics and Gynaecology, Dijkzigt Hospital, Rotterdam; C. Jansen, L. van Os, Department of Obstetrics and Gynaecology, Diaconessenhuis, Voorburg. Norway: T. Abyholm, T. Tanbo, L. Henriksen, Kvinnekliniken Rikshopitalet, Oslo; J. Kahn, A. Sunde, L. Reinertsen, Kvinnekliniken Regionsykehuset i Trondheim, Trondheim. Spain: P. Barri, P. Cabrero, M. Torello, Servicio de Reproducción Humana, Instituto Dexeus, Barcelona; J. Calaf Alsina, Carabela La Niña No. 24, Barcelona. Sweden: L. Hamberger, L. Nilsson, C. Bergh, A. Strandell, B. Josefsson, Kvinnokliniken, Sahlgrenska Sjukhuset, Göteborg; T. Bergh, Department of Obstetrics and Gynaecology, Uppsala University, Akademiska Fjukhuset, Uppsala; J. Harlin, Department of Obstetrics and Gynaecology, Karolinska Hospital, Stockholm; N.O. Sjöberg, L. Hägglund, F. Ploman, Department of Obstetrics and Gynaecology, University of Lund, Malmö. Switzerland: M. Birkhäuser, Universitäts-Frauenklinik und Kantonales Frauenhospital, Bern. United Kingdom: M. Ohbrai, Norths Staffs Maternity Hospital, Harpfields, Stoke-on-Trent, Staffordshire; J. Ginsburg, Department of Endocrinology, The Royal Free Hospital, Pond Street, London; S. Franks, Department of Obstetrics and Gynaecology, St. Mary's Hospital Medical School, London; R.G. Forman, S.A. Adeaga, Department of Obstetrics and Gynaecology, Guy's Hospital, London; B. Lieberman, P. Buck, P. Matson, F. Hamer, A. Ratcliffe, J. Bent, G. Horne, Regional IVF Unit, St. Mary's Hospital, Manchester; R.W. Shaw, S. Vine, Department of Obstetrics and Gynaecology, University of Wales College of Medicine, Cardiff; C.P. West, H. Hillier, The Assisted Conception Unit, Royal Infirmary of Edinburgh, Edinburgh; R.M.L. Winston, N. Reddy, G. Atkinson, A. Huodono, Hammersmith Royal Postgraduate Medical School, Institute of Obstetrics and Gynaecology, London and IVF Clinic, Royal Masonic Hospital, London. NV Organon, Oss The Netherlands: B. de Blank, W. de Boer, H. Coelingh Bennink, S. Driessen, F. Eikelboom, J. Geelen, P. Janssen, H. Joosten, R. de Leeuw, B. Mannaerts, F. van Meel, A. de Meere, F. Metsers, J. Mulders, H.J. Out, R. Paulussen, F. Rombout, A. Skrabanja, I. van de Veerdonk, R. Verbeeten, J. Verhoeven and many others.

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Human Reproduction vol.10 no.10 pp.2534–2540, 1995

A prospective, randomized, assessor-blind, multicentre study comparing recombinant and urinary follicle stimulating hormone (Puregon versus Metrodin) in in-vitro fertilization

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Urinary follicle stimulating hormone (FSH) is being used for the treatment of human infertility. Recently, FSH manufactured by means of recombinant DNA technology with a much higher purity (>99%) has become available. A prospective, randomized, assessor-blind, multicentre (n = 18) study was conducted in infertile women undergoing in-vitro fertilization comparing recombinant FSH (Org 32489, Puregon®) and urinary FSH (Metrodin®). Eligible subjects were randomized (recombinant versus urinary FSH = 3:2) and pretreated with buserelin for pituitary suppression. FSH was given until three or more follicles with a diameter of at least 17 mm were seen. After oocyte retrieval, fertilization routines were applied according to local procedures. No more than three embryos were replaced. In all, 585 subjects received recombinant FSH and 396 urinary FSH. Significantly more oocytes were retrieved after recombinant FSH treatment (mean adjusted for centre 10.84 versus 8.95, $P < 0.0001$). Ongoing pregnancy rates per attempt and transfer in the recombinant FSH group were 22.17 and 25.97% respectively, and in the urinary FSH group, 18.22 and 22.02% respectively (not significant). Ongoing pregnancy rates including pregnancies resulting from frozen–thawed embryo cycles were 25.7% for recombinant and 20.4% for urinary FSH (P = 0.05). Compared to urinary FSH, the total dose of FSH was significantly lower with recombinant FSH (2138 versus 2385 IU, $P < 0.0001$) in a significantly shorter treatment period (10.7 versus 11.3 days, $P < 0.0001$). No clinically relevant differences between recombinant and urinary FSH were seen with respect to safety variables. It is concluded that recombinant FSH (Puregon) is more effective than urinary FSH in inducing multifollicular development and achieving an ongoing pregnancy.

Key words: IVF/ovarian stimulation/Puregon/recombinant FSH

Introduction

For >30 years, human menopausal gonadotrophins (HMG) have been applied in the treatment of human infertility. Clinical applications include ovulation induction in clomiphene-resistant anovulatory women and ovarian stimulation in assisted reproduction techniques, e.g. in-vitro fertilization (IVF) (Breckwoldt and Zahradnik, 1991). Most HMG preparations contain either equal amounts of follicle stimulating hormone (FSH) and luteinizing hormone (LH) activity (FSH/LH ratio = 1) or mainly FSH activity with minor amounts of LH activity (urofollitrophin, FSH/LH ratio ≥60). The production of these hormones depends on the collection of huge amounts of urine. The use of urine sources implies limited product consistency and purity (1–5%).

Recently, FSH has been manufactured by means of recombinant DNA technology using a Chinese hamster ovary (CHO) cell line transfected with the genes encoding human FSH (Van Wezenbeek *et al.*, 1990). The final product (Org 32489, Puregon®) is purified up to 99% purity, does not contain any LH activity and is very similar to natural FSH (Hård *et al.*, 1990), although small differences in oligosaccharide moieties and isohormone composition are present.

Clinical experiences with recombinant FSH indicate the potential of the compound to induce follicular growth, and pregnancy can be achieved (Devroey *et al.*, 1994). In this paper, a multicentre trial is described evaluating the efficacy and safety of recombinant FSH to achieve ovarian stimulation in infertile women undergoing IVF, in comparison with urinary FSH.

Materials and methods

Patients

Between March 1992 and August 1993, infertile female subjects were recruited at 18 different IVF centres throughout Europe (see Acknowledgements section). The aim was to include 1000 patients. Inclusion criteria were as follows: patients had to be 18–39 years of age at the time of screening; have a cause of infertility which was potentially solvable by IVF; a maximum of three previous IVF or other assisted reproduction attempts in which oocytes were collected at least once; normal ovulatory cycles with a mean length of between 24 and 35 days and an intra-individual variation of plus or minus 3 days (but never outside the 24–35 days range); good physical and mental health; and a body weight 80–130% of the ideal body weight (adapted from the Metropolitan Life Insurance Company Tables).

Exclusion criteria were: infertility caused by endocrine abnormalities such as hyperprolactinaemia, polycystic ovary syndrome, and absence of ovarian function; male infertility as defined by $<10\times10^6$ spermatozoa/ml and/or <40% normal morphology and/or <40% normal motility; any ovarian and/or abdominal abnormality that would interfere with adequate ultrasound investigation; hypertension (sitting diastolic blood pressure >90 mm Hg and/or systolic blood

pressure >150 mm Hg); chronic cardiovascular, hepatic, renal, or pulmonary disease; a history of (within 12 months) or current abuse of alcohol or drugs; administration of non-registered investigational drugs within 3 months prior to screening. When all criteria were met, the subject was considered to be eligible. The study was approved by the Ethics Committee of each local hospital. All subjects gave written informed consent. This investigation was performed according to the Declaration of Helsinki and the European Community note on Good Clinical Practice for trials on medicinal products in the European Community (CPMP Working Party on Efficacy of Medicinal Products, 1990).

Study design

This was a randomized, assessor-blind, prospective, multicentre study comparing recombinant human FSH (Org 32489, Puregon®, NV Organon, Oss, The Netherlands, batch numbers CP 091134 and 091077) and urinary FSH (urofollitropin, Metrodin®, Ares-Serono, Switzerland, batch numbers CP 092139, 093057, 092047, 091163). The objective of the study was to assess the efficacy and safety of recombinant FSH in relation to urinary FSH for the induction of ovarian stimulation in infertile pituitary-suppressed subjects undergoing IVF. Eligible subjects were randomized by receiving a subject number from a randomization list corresponding with patient boxes in which the medication was kept. The randomization procedure included a ratio between recombinant and urinary FSH of 3:2. All centres followed an identical clinical protocol and used standardized case report forms.

Pituitary down-regulation started on the first day of the menstruation by means of intranasal buserelin (Suprecur®, Hoechst, Germany). The initial dose was 4×150 µg daily. When suppression was not achieved (serum oestradiol >200 pmol/l) after 14 days, the dose was doubled (4×300 µg daily). The buserelin intake was sustained throughout the FSH treatment. The FSH dose for the first 4 days was 150 or 225 IU (two or three ampoules i.m.). Afterwards, the dose was adjusted according to follicular development as assessed by ultrasound scanning. Since, for technical reasons, recombinant FSH was supplied in vials and urinary FSH in ampoules, a double-blind design was not feasible. Instead, an assessor-blind design was chosen in which preparation and administration of the medication was done by a study co-ordinator who took no part in any decision concerning the FSH dose during treatment. When at least three follicles ≥17 mm were present, 10 000 IU of human chorionic gonadotrophin (HCG, Pregnyl®, NV Organon, The Netherlands) was given i.m. to induce ovulation. Oocyte retrieval, fertilization procedures and embryo transfer were done according to the local standards. A maximum of three embryos was transferred. Luteal support was given during at least 2 weeks and included minimally three injections of 1500 IU HCG or at least 50 mg of progesterone daily i.m. or 400 mg progesterone daily intravaginally.

End-points

The primary outcome variables were the number of oocytes retrieved, and ongoing pregnancy rate per attempt and transfer as assessed by ultrasound scanning at least 12 weeks following embryo transfer.

Secondary variables included number of follicles ≥15 mm and ≥17 mm on the day of HCG administration, length of FSH treatment, total dose, serum concentrations of FSH and oestradiol on the day of administering HCG, number of mature oocytes recovered, number of high quality embryos, implantation rate, clinical pregnancy rates per attempt and transfer. Implantation rate was defined as the number of vital fetuses as assessed by ultrasound at least 12 weeks after embryo transfer, divided by the number of embryos transferred for each subject. The definition of a clinical pregnancy included miscarriages with or without proof of a vital fetus.

Fertilization and cleavage rates are not reported due to the heterogeneity in IVF routines across the centres.

The main safety parameters were the incidence of ovarian hyperstimulation syndrome (OHSS) and the development of anti-FSH antibodies and anti-CHO cell-derived protein antibodies. Also, common laboratory parameters were compared before and after treatment. These parameters included routine blood biochemistry as sodium, potassium, chloride, bicarbonate, phosphorus, calcium, glucose, urea, creatinine, alkaline phosphatase, alanine amino transferase, aspartase amino transferase, lactic dehydrogenase, total bilirubin, total protein, albumin; haematology parameters included haemoglobin, haematocrit, erythrocytes, leukocytes plus differentiation; urinalysis included quantitative estimation of pH and qualitative estimations of protein, acetone, glucose, and haemoglobin.

Assessments

At screening, the medical history was obtained and a physical examination was performed. Routine blood biochemistry, haematology and urinalysis were done and the following endocrinological parameters were measured: serum oestradiol, FSH, LH, progesterone, testosterone, prolactin, and dehydroepiandrosterone sulphate. An ultrasound scan was done to exclude ovarian abnormalities. Sperm analysis of the partner took place and was repeated at the time of fertilization.

Serum oestradiol concentrations were measured to ensure optimal pituitary suppression prior to the first FSH injection. Serum FSH, LH, oestradiol and progesterone were measured on the first day of FSH treatment and on the day of HCG administration. In between, assessments of serum oestradiol and LH were done on a regular basis. Frequent ultrasound scans were made to monitor follicular growth.

Spare serum samples for the determination of anti-FSH and anti-CHO-cell derived protein antibodies were taken before and after treatment. Routine blood biochemistry, haematology and urinalysis were repeated as soon as possible after FSH treatment had ended.

Classification of oocytes as either mature or immature and embryos as type 1, 2, 3, or 4 was done according to previously published criteria (Staessen *et al.*, 1989). Type 1 and 2 were considered to be high quality embryos.

Assays

Antibody assay

Blood samples processed to serum taken before and after FSH treatment were sent to NV Organon, The Netherlands for central determination of anti-FSH and anti-CHO cell-derived protein antibodies.

Anti-FSH antibodies. The presence of specific antibodies against human FSH was assessed by a semi-quantitative radioimmunoassay in duplicate. In short, ^{125}I-labelled recombinant FSH was allowed to react with antibodies present in the sample. The immune-complexes formed were subsequently precipitated with polyethylene glycol (PEG 8000). After removal of the supernatant, bound radioactivity in the pellet was quantified. A calibration curve with human anti-FSH antibodies that would allow quantitative determination of the serum anti-FSH antibody concentration could not be established, since there is no representative standard human anti-FSH antibody preparation available. Therefore, all values were expressed as percentage of the total amount of tracer added in the assay and were corrected for the non-specific binding. Clinically relevant antibody titres were defined as those yielding a binding percentage of >25%.

Anti-CHO cell-derived protein antibodies. The occurrence of antibodies against proteins from the CHO cell line was assessed by a

semi-quantitative enzyme-immunoassay. In short, CHO cell-derived proteins were coated to the wall of 96-well microtitre plates. Antibodies in the sample were allowed to bind to the solid-phase-coated CHO cell-derived proteins, where they were then detected with a horseradish peroxidase coupled second antibody [goat antihuman immunoglobulin (IgG)]. The end product of the enzyme reaction was quantified spectrophotometrically at 450 nm, corrected for the optical density at 690 nm. Each analytical run included a series of six concentrations in human serum of the IgG fraction of a rabbit polyclonal antiserum against CHO cell-derived proteins as positive control. The CHO cell-derived proteins used to obtain this antiserum and the CHO cell-derived proteins applied in the assay were purified from the culture supernatant of a mock-transfected CHO cell line.

Other assays

Sperm analysis and measurement of blood biochemistry, haematology, urinalysis and endocrinological parameters were done at the local hospital according to local standards. Follicular size was measured with local ultrasound equipment and a vaginal probe.

Sample size

Power calculations were performed in order to assess the magnitude of treatment effects capable of detection in this large study, and were based on efficacy data of 1000 subjects, assuming that at least 850 subjects had an oocyte retrieval and embryo transfer (Dupont and Plummer, 1990). When testing at the customary 5% significance level (two-sided), and assuming an SD of 6, a difference of 1.2 oocytes in the two treatment groups would have been detected statistically with a probability of 80%. With respect to dichotomous variables such as pregnancy, by assuming a pregnancy rate of 15% per attempt and 18% per transfer for one treatment group, a value per attempt (and transfer) as small as 9% (11%) or as large as 22% (26%) for the second group would have been detected statistically with an 80% probability, using a two-sided χ^2 test, again at the 5% significance level. Therefore, the size of the study ensured that fairly modest treatment effects would have been detected with a high probability.

Statistical analysis

For ordinal data a general parametric approach (Whitehead and Whitehead, 1991) of combining individual centre results was applied and used for communication of results and eventual analysis. For binary data (pregnancy outcome) the Mantel–Haenszel test statistic extended for multiple centres was used. In both cases the combination of centre results was expressed as means adjusted for centre and approximate confidence intervals (CI) were calculated based on the normal distribution.

All analyses were done on an intent-to-treat basis, including all subjects who received at least one ampoule of FSH. The main advantages of this rule were that more patients were available for final analysis of efficacy and that it more closely reflected how physicians evaluate a therapeutic agent in the clinical setting, outside an experimental control.

Results

Patients

A total of 1027 subjects (recombinant FSH: n = 615, urinary FSH: n = 412) was randomized, 1007 (recombinant FSH: n = 602, urinary FSH: n = 405) started buserelin pretreatment and 981 (recombinant FSH: n = 585, urinary FSH: n = 396) started FSH treatment. The number of subjects treated with FSH per centre was 10–146 (mean 54.5).

Both treatment groups were comparable in demographic and infertility characteristics (Table I). The main cause of infertility was tubal disease (64.4 and 64.1% for recombinant versus urinary FSH respectively). The mean duration of infertility for recombinant and urinary FSH was 6.3 and 6.1 years respectively.

Table I. Demographic and infertility characteristics

Characteristic	Recombinant FSH (n = 585)	Urinary FSH (n = 396)
Mean age (years)	32.2	32.3
Mean weight (kg)	61.3	61.2
Mean height (cm)	164.4	164.3
Number (%) of subjects with cause of infertility		
Tubal disease	377 (64.4)	254 (64.1)
Endometriosis	45 (7.7)	30 (7.6)
Tubal disease + endometriosis	23 (3.9)	15 (3.8)
Unknown	117 (20.0)	79 (19.9)
Other	23 (3.9)	18 (4.5)
Mean duration of infertility (years)	6.3	6.1
Number (%) of subjects with primary infertility	259 (44.3)	174 (43.9)
Number (%) of subjects with secondary infertility	326 (55.7)	222 (56.1)

Primary efficacy parameters

The results of the main efficacy parameters are given in Table II. In the recombinant FSH group, a mean number (adjusted for centre) of 10.84 oocytes was recovered, compared to 8.95 in the urinary FSH group. The difference of 1.89 was highly significant (P <0.0001; 95% CI 1.2–2.6). The mean number of oocytes recovered across the centres ranged from 7.2 to 16.4 for recombinant FSH and from 4.0 to 12.8 for urinary FSH, the differences varying from 0.62 to 5.50 oocytes. In all centres, more oocytes were retrieved after recombinant FSH treatment (Figure 1).

Ongoing pregnancy rates per attempt and transfer and adjusted for centre were 22.17 and 25.97% respectively for the recombinant FSH group, and 18.22 and 22.02% respectively, in the urinary FSH group. Until August 1994, 117 and 73 subjects in the recombinant and urinary FSH group respectively, subsequently underwent a natural cycle during which frozen–thawed embryos were replaced, resulting in 17 ongoing pregnancies in the recombinant FSH group and five in the urinary FSH group. A second 'frozen embryo' cycle was done in 26 and 15 women, which resulted in seven additional pregnancies: five in the recombinant and two in the urinary FSH group. Eight women had a third frozen embryo cycle and two subjects a fourth, which did not result in ongoing pregnancies. The mean number of embryos transferred in the frozen embryo cycles was 2.1 for both groups. In total, 22 additional pregnancies were obtained in the recombinant FSH group, and seven in the urinary FSH group, resulting in cumulative ongoing pregnancy rates (adjusted for centre) of 25.7 and 20.4% in favour of recombinant FSH (P = 0.05).

Secondary efficacy parameters

Results of the secondary parameters are given in Table III. On the day of HCG, significantly more follicles ≥15 mm were seen in the recombinant FSH group (n = 7.49, mean adjusted

Table II. Results on main parameters

Parameter	Mean adjusted for centre		Recombinant minus urinary FSH		
	Recombinant FSH	Urinary FSH	Difference	SE	95% CI
No. of oocytes recovered	10.84	8.95	1.89	0.37	1.2–2.6 ($P < 0.0001$)
Ongoing pregnancy rate (%) per attempt	22.17	18.22	3.95	2.59	−1.1–9.0 NS
Ongoing pregnancy rate (%) per transfer	25.97	22.02	3.95	3.01	−1.9–9.8 NS

CI = confidence interval.
NS = not significant.

for centre), compared to the urinary FSH group (n = 6.67, P = 0.0002, 95% CI of difference: 0.4–1.2). Figure 2 demonstrates that development of large (≥15 mm) follicles began to diverge between the groups after 5–6 days of FSH treatment. This higher number of follicles was associated with a significantly increased maximum serum oestradiol in the recombinant FSH group (mean adjusted for centre 6084 versus 5179 pmol/l, P <0.0001). On the day of HCG, FSH concentrations were significantly higher in the urinary FSH group (12.1 versus 11.5 IU/l, P = 0.03). A significantly lower total dose of recombinant FSH (mean adjusted for centre 2138 versus 2385 IU, P <0.0001) were needed in an also significantly shorter treatment period (10.7 versus 11.3 days, P <0.0001) compared to the urinary FSH group. There was no linear relationship between the number of ampoules administered and number of oocytes collected.

Mean LH concentrations after down-regulation before start of the FSH treatment were 1.6 and 1.7 IU/l, and on the day of HCG administration 1.2 and 1.3 IU/l in the recombinant and urinary FSH groups respectively.

After oocyte retrieval, more mature oocytes (difference 1.8, P <0.0001; 95% CI 1.1–2.4) were recovered and more high quality embryos (difference 0.5, P = 0.003; 95% CI 0.2–0.8) were obtained in the recombinant FSH group.

No significant differences between recombinant and urinary FSH were seen in the number of follicles ≥17 mm on the day of HCG, the implantation rate and the clinical pregnancy rates per attempt and transfer (see Table III).

The mean number of oocytes with two pronuclei was 6.8 and 5.6 in the recombinant and urinary FSH groups respectively, as assessed 12–18 h after incubation with semen. Oocytes with three or more pronuclei were seen in 208 subjects (38.2%) in the recombinant FSH group, compared to 117 (32.4%) in the urinary FSH group. A mean number of 2.58 and 1.81 embryos were frozen in the recombinant and urinary FSH groups respectively.

Cycle cancellations

In all, 152 patients started FSH treatment but did not have an embryo transfer (recombinant FSH: n = 85, 14.5%; urinary FSH: n = 67, 16.9%; not significantly different). Low ovarian response was reported in 27 subjects in the recombinant FSH group (4.6%) and in 30 in the urinary FSH group (7.6%). The risk of OHSS was the reason for cancellation in 12 recombinant FSH-treated subjects (2.1%) and six urinary FSH-treated subjects (1.5%). Unsuccessful fertilization was the reason for premature discontinuation in 29 subjects (5.0%) in the recombinant FSH group, compared to 16 (4.0%) in the urinary FSH group.

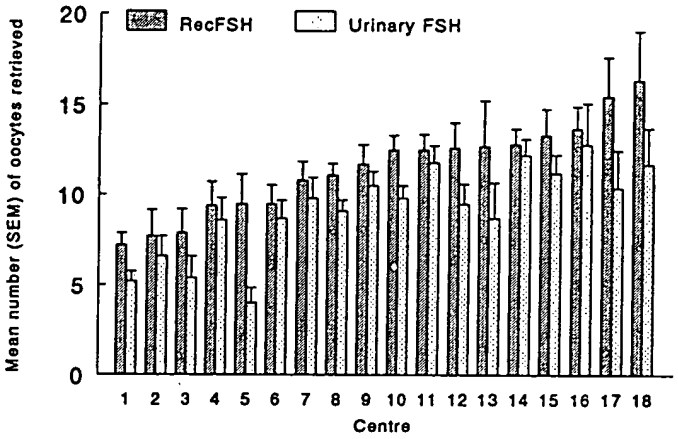

Figure 1. Mean (+ SEM) number of oocytes retrieved per centre. FSH = follicle stimulating hormone. Rec = recombinant.

Safety

OHSS leading to hospitalization was seen in 19 out of 585 recombinant FSH-treated subjects (3.2%) compared with eight out of 396 urinary FSH-treated subjects (2.0%, not significantly different). In 545 recombinant FSH-treated and 353 urinary FSH-treated subjects spare serum samples could be assessed for the presence of anti-FSH and anti-CHO cell-derived protein antibodies. No significant rises of serum antibody concentrations were found. Clinically relevant changes from base line of routine blood biochemistry, haematology and urinalysis were not detected.

Discussion

To our knowledge, this is the largest prospective, randomized clinical trial ever performed in IVF. In total, 585 subjects received recombinant FSH (Puregon®) and 396 urinary FSH (Metrodin®) for ovarian stimulation. The aim of stimulation was to increase the number of oocytes for assisted reproduction. Therefore, the number of oocytes retrieved was chosen as

Table III. Results on secondary parameters

Parameter	Mean adjusted for centre		Recombinant minus urinary FSH		
	Recombinant FSH	Urinary FSH	Difference	SE	95% CI
No. of follicles ≥15 mm on day of HCG	7.49	6.67	0.81	0.22	0.4–1.2 (P = 0.0002)
No. of follicles ≥ 17 mm on day of HCG	4.61	4.38	0.23	0.14	−0.0–0.5 NS
Maximum serum oestradiol (pmol/l)	6084	5179	905	210	494–1317 (P < 0.0001)
Serum FSH on day of HCG (IU/l)	11.5	12.1	−0.6	0.26	−1.1 to −0.1 (P = 0.03)
Total no. of ampoules used	28.5	31.8	−3.3	0.62	−4.5 to −2.1 (P < 0.0001)
Treatment length (days)	10.7	11.3	−0.6	0.13	−0.9 to −0.3 (P < 0.0001)
No. of mature oocytes recovered	8.55	6.76	1.79	0.33	1.1–2.4 (P < 0.0001)
No. of high quality embryos	3.11	2.61	0.50	0.17	0.2–0.8 (P = 0.003)
Implantation rate (%)	0.11	0.09	0.01	0.02	−0.02–0.05 NS
Clinical pregnancy rate (%) per attempt	29.29	25.30	3.99	2.88	−1.6–9.6 NS
Clinical pregnancy rate (%) per transfer	34.30	30.46	3.84	3.31	−2.6–10.3 NS

CI = confidence interval.
NS = not significant.
HCG = human chorionic gonadotrophin.
FSH = follicle stimulating hormone.

one of the main efficacy parameters in this study. Ovarian stimulation was continued until there was evidence of adequate multiple follicular development (at least three follicles ≥17 mm in diameter). The fact that the treatment groups did not differ significantly with respect to the number of follicles ≥17 mm on the day of HCG indicated that the stimulation procedures were carried out in a similar way. However, a significantly higher number of oocytes was retrieved in the recombinant FSH group. Despite the large well-known differences between centres in overall number of oocytes retrieved, this finding was consistently in favour of recombinant FSH throughout all centres (Figure 1). Accordingly, a larger cohort of follicles was recruited in these subjects, as illustrated by the significantly higher number of follicles ≥15 mm in the recombinant FSH group seen on the last ultrasound before oocyte retrieval. The significantly higher maximum serum oestradiol concentration in the recombinant FSH group is most likely a reflection of this larger number of follicles. Interestingly, overall concentrations of immunoreactive FSH on the day of HCG were slightly but significantly lower in the recombinant FSH group, even though different types of FSH assays were applied which increased the overall variability. This might be related to the significantly lower amount of recombinant FSH administered and the significantly shorter treatment period in this group. It also illustrates that FSH concentrations based on immunoassay measurements have only limited value in assessing the true potency, since they only reflect the number of circulating FSH molecules but not their actual biological activity.

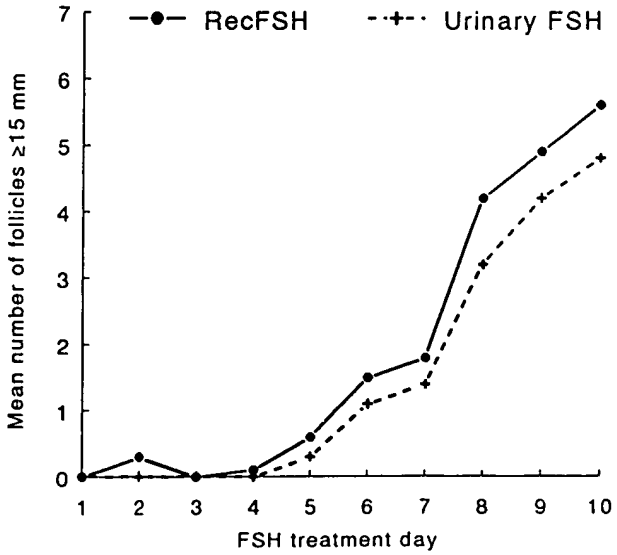

Figure 2. Mean number of follicles ≥15 mm related to the follicle stimulating hormone (FSH) treatment day. Rec = recombinant.

According to pharmacopeial requirements (Council of Europe, 1986), the FSH activity of a batch is calibrated in the in-vivo Steelman–Pohley rat assay, against an International Standard Preparation (Steelman and Pohley, 1953). This rat model is apparently not valid to predict clinical activity in the human, given the differences we found using nominally equal preparations with the same declared content, namely 75 IU in-vivo bioactivity per ampoule.

Possible factors which might explain the higher potency of recombinant FSH compared to urinary FSH include subtle differences at the level of the oligosaccharide moieties of the molecules, differences in isohormone composition (Matikainen *et al.*, 1994), or the proteinaceous contaminants in the urinary product inhibiting FSH action and the pharmaceutical formulation. Further research is needed to elucidate the influence of these factors on the clinical efficacy of gonadotrophin preparations. Not all recombinant FSH products give identical results with IVF, or are superior to urinary FSH (recombinant human FSH study group, 1995). In fact, in that study oestradiol levels at the day of HCG administration were significantly lower in the recombinant FSH group. This surely suggests that differences between various recombinant FSH preparations exist.

With respect to the other main efficacy parameter, ongoing pregnancy rate, no statistically significant difference between the recombinant and urinary FSH groups was found. This is to be expected since the significant treatment differences in favour of recombinant FSH, such as the higher number of oocytes retrieved and the larger number of high quality embryos, are basically nullified since both groups 'restarted' treatment at an equal position at the moment of transfer of a fixed maximum number of embryos. In both treatment groups a mean of 2.4 embryos were replaced. However, differences in ongoing pregnancy rates including 'frozen embryo' cycles reached statistical significance in favour of recombinant FSH ($P = 0.05$). This might be due to the availability of better quality embryos after recombinant FSH treatment, next to the obvious reason that merely the presence of more embryos ultimately will lead to more pregnancies.

The most important side-effect of gonadotrophin treatment in ovarian stimulation is the occurrence of OHSS. This possibly life-threatening condition is characterized in its most serious forms by ascites, haemoconcentration, coagulation and electrolyte disorders and extreme ovarian enlargement (Rizk and Smitz, 1992). Despite the higher number of follicles recruited and the increased serum oestradiol concentration on the day of HCG administration, which are both risk factors for the development of the syndrome, its incidence was not statistically significantly higher after recombinant FSH treatment. However, the incidence of severe hyperstimulation requiring hospitalization is so low that the power of the study would be insufficient to detect a significant difference of 2%. Therefore, given the higher potency of recombinant FSH, careful monitoring to prevent the occurrence of this syndrome is essential.

Recombinant and natural FSH differ slightly at the level of carbohydrate moieties (Hård *et al.*, 1990) and potentially, minute amounts of host cell-originating contaminations might be present in the recombinant preparation. To investigate the immunogenic characteristics, and to rule out any risk, antibody development against FSH and CHO cell-derived proteins was assayed. Antibody formation was not seen in any of the recombinant FSH-treated patients.

In conclusion, this study has demonstrated that recombinant FSH (Puregon®) is more efficacious than urinary FSH (Metrodin®) as assessed by the number of oocytes retrieved. Pregnancy rates including frozen embryo cycles were significantly higher after recombinant FSH treatment. Recombinant and urinary FSH are equally safe.

Acknowledgements

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Received on October 4, 1994; accepted on July 20, 1995

EXHIBIT 1

US005270057A

United States Patent [19]

de Meere et al.

[11] Patent Number: 5,270,057

[45] Date of Patent: Dec. 14, 1993

[54] STABILIZED GONADOTROPIN CONTAINING PREPARATIONS

[75] Inventors: Andreas L. J. de Meere, Waspik; Marinus A. De Ruiter, Oss, both of Netherlands

[73] Assignee: Akzo N.V., Arnhem, Netherlands

[21] Appl. No.: 914,227

[22] Filed: Jul. 13, 1992

Related U.S. Application Data

[63] Continuation of Ser. No. 672,509, Mar. 20, 1990, abandoned.

[30] Foreign Application Priority Data

Mar. 20, 1990 [EP] European Pat. Off. 90.200665.9

[51] Int. Cl.[5] A61K 9/14; A61K 37/38

[52] U.S. Cl. 424/499; 514/2; 514/8; 514/21; 514/22; 530/398; 530/399; 530/850

[58] Field of Search 424/499; 514/2, 8, 21, 514/23; 530/398, 399, 850–853

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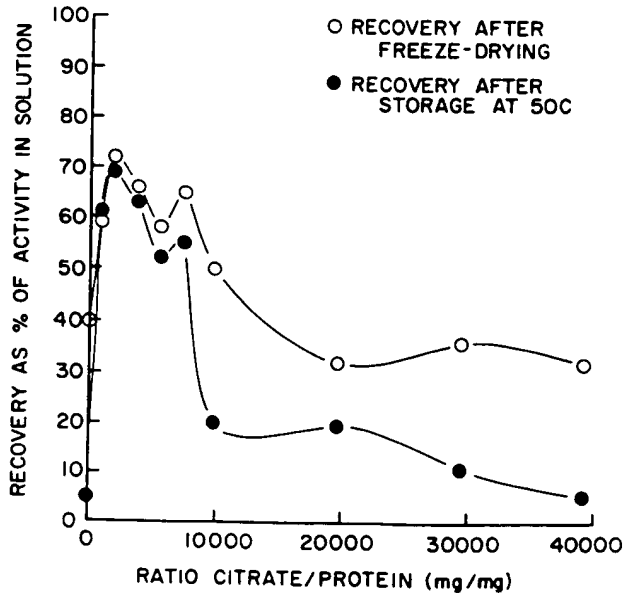
Attorney, Agent, or Firm—William M. Blackstone

[57] ABSTRACT

Disclosed are lyophilized gonadotropin containing preparations containing a dicarboxylic acid salt stabilizer. The particular proteins (e.g. LH, TSH, FSH, or HCG) are in admixture with, and at least partially capable of stabilization by, the particular stabilizer in lyophilized form. The preparations contain a sufficient amount of dicarboxylic acid salt to stabilize the protein in freeze-dried form for a desired time at a desired temperature. Typical dicarboxylic acid salts disclosed are the salts of citric, tartaric, and aspartic acids. The preparations preferably include a non-reducing disaccharide to increase the collapse temperature of the solution to be lyophilized. Methods of making the preparations in lyophilized form and the resulting injectable preparations are also disclosed.

7 Claims, 2 Drawing Sheets



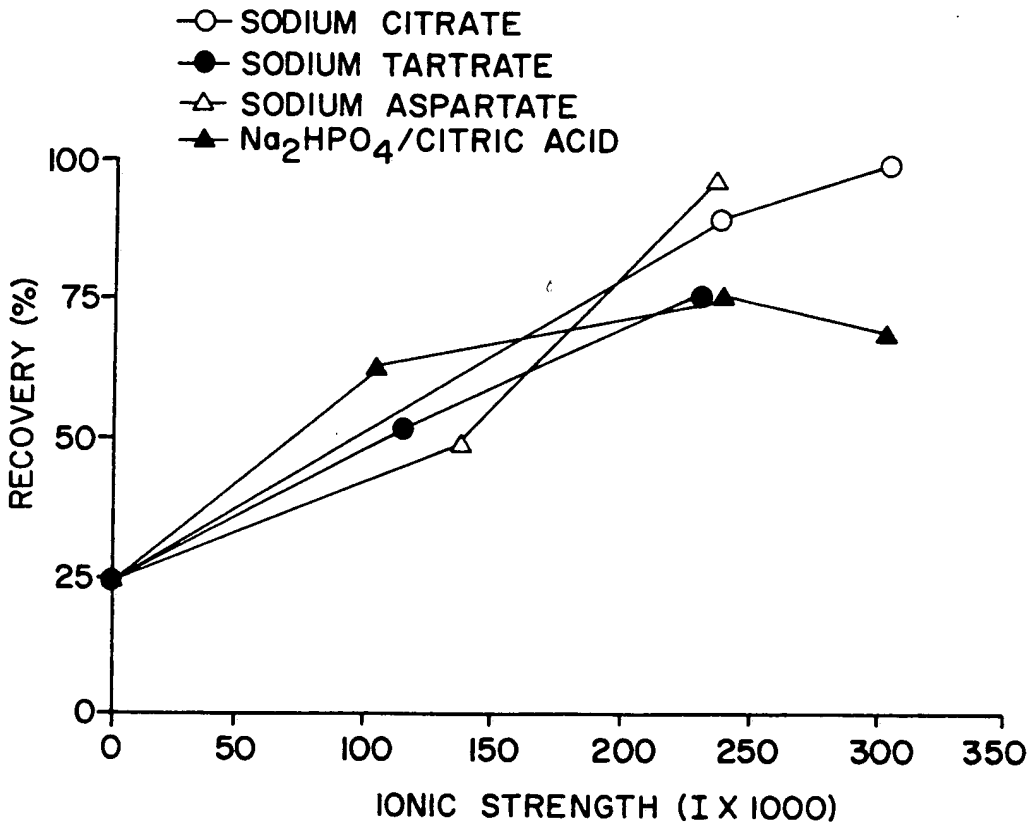

SODIUM CITRATE
SODIUM TARTRATE
SODIUM ASPARTATE
Na_2HPO_4/CITRIC ACID
RECOVERY (%)
100
75
50
25
0
0
50
100
150
200
250
300
350
IONIC STRENGTH (I X 1000)

FIG. 1

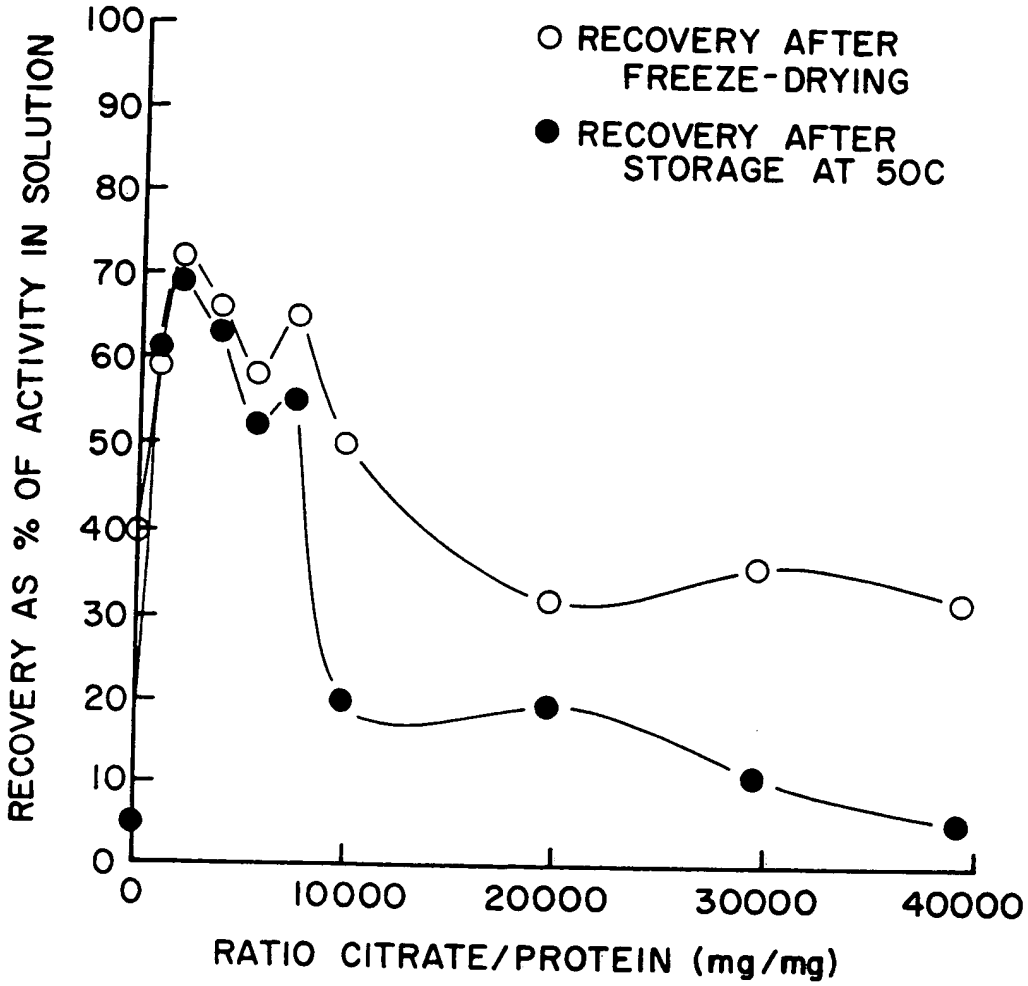

RECOVERY AS % OF ACTIVITY IN SOLUTION
100
90
80
70
60
50
40
30
20
10
0
0
10000
20000
30000
40000
RATIO CITRATE/PROTEIN (mg/mg)
O RECOVERY AFTER FREEZE-DRYING
● RECOVERY AFTER STORAGE AT 50C

FIG. 2

STABILIZED GONADOTROPIN CONTAINING PREPARATIONS

This is a continuation of application Ser. No. 07/672,509, filed Mar. 20, 1991, now abandoned.

BACKGROUND OF THE INVENTION

1. Field

This invention relates to pharmaceutical compositions generally, and to stabilized gonadotropin containing preparations specifically.

2. State of the Art

Relatively pure gonadotropin preparations are commercially available. For example, compositions containing naturally derived human menopausal gonadotropin ("HMG") and naturally derived human chorionic gonadotropin ("HCG") are available as freeze-dried preparations under the trade designations "Humegon" and "Pregnyl," respectively, from Organon International, bv of Oss, NL. Pregnant mare gonadotropin is also available in a freeze dried form from the same company.

A bulking agent, e.g. mannitol, is added to these preparations before lyophilization. They do not require the addition of a stabilizer to ensure an adequate shelf-life. Evidently whatever natural contaminants remain after the purification process act to stabilize the preparations in freeze-dried form.

Recently however, with the advent of more effective production and purification techniques, preparations of certain very pure gonadotropins are insufficiently stable. They degrade in a relatively short time, losing activity. In order to prevent or slow down this degradation, attempts were made to freeze-dry (lyophilize) the preparations. Lyophilization has only been partially successful however.

A need exists for a gonadotropin containing pharmaceutical preparation which is stable over a sufficiently long period of time for the product to be manufactured, shipped, and stored prior to use. The need is especially great for a stable preparation containing more than one gonadotropin.

SUMMARY OF THE INVENTION

Generally, the invention includes a gonadotropin containing lyophilized protein preparation which contains a dicarboxylic acid salt stabilizer. "Dicarboxylic acid," as used herein, means an organic acid having two or more carboxylic acid moieties (e.g HOOC—R—COOH). The gonadotropin will be in admixture with, and at least partially capable of stabilization by, the particular stabilizer in lyophilized systems. The preparation will contain a sufficient amount of dicarboxylic acid salt to stabilize the gonadotropin in its freeze-dried form for a desired time at a desired temperature.

Typical dicarboxylic acid salts are salts of citric acid, tartaric acid, aspartic acid, or mixtures of these acids. The gonadotropin or gonadotropin derivatives (as used herein "gonadotropins") will typically be proteins such as follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH), human chorionic gonadotropin (HCG), or luteinizing hormone (LH). The preparation can further include a non-reducing disaccharide, such as sucrose or trehalose.

The invention also includes a method of stabilizing an essentially pure gonadotropin, in lyophilized form, which method involves mixing the gonadotropin, in solution, with a sufficient amount of a dicarboxylic acid salt to stabilize the protein in the lyophilized form, and then freeze-drying the resulting solution to form a stabilized lyophilisate of the gonadotropin.

The invention further includes the reconstituted injectable preparation made from the lyophilisate. The injectable preparation consists essentially of aqueous solution of water for injection, the gonadotropin, a non-reducing sugar, an anti-adsorption agent, and the dicarboxylic acid salt.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph depicting the correlation between ionic strength (X1000) versus the amount of recovery of recombinant FSH activity after 1 month at 60° C. using various stabilizers.

FIG. 2 is a graph depicting the effect of citrate/-protein ratio (weight/weight) on recovery of HCG activity.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

A. Gonadotropins

Preferred gonadotropins are FSH, TSH, HCG, LH, derivatives and mixtures thereof, with or without other protein components. Follicle stimulating hormone, thyroid stimulating hormone, human chorionic gonadotropin, and luteinizing hormone are all chemically related proteins which consist of α and β subunits. The α subunits of these proteins are identical or nearly so.

Follicle stimulating hormone is a hormonal glycoprotein of the anterior pituitary required for normal reproductive function. Follicle stimulating hormone has been used to stimulate development of ovarian follicles for in vitro fertilization, and has also been used clinically to stimulate follicular maturation in anovulatory women with chronic anovulatory syndrome or luteal phase deficiency. Follicle stimulating hormone may be at least partially isolated from natural sources, such as human urine. Recombinant follicle stimulating hormone and/or LH may be prepared as described in Keene et al "Expression of Biologically Active Human Follitropin in Chinese Hamster Ovary Cells," *The Journal of Biological Chemistry*, Vol. 264, pp. 4769–4775 (25 March 1989), the contents of which are incorporated by this reference. As used herein, a gonadotrophin, for example follicle stimulating hormone (FSH), includes the compound's analogs, and its recombinant, natural, deglycosylated, unglycosylated, modified glycosylated, and other forms.

The most preferred gonadotropin is FSH produced by recombinant DNA techniques (rFSH), either alone or in a lyophilisate with LH or HCG. FSH purified from natural sources is generally only partially purified. The impurities seem to act to stabilize it somewhat. With rFSH, however the impurities are not present, and thus the FSH is more susceptible to rapid degradation and freeze-drying losses. Doses of FSH range from 60 to 1500, especially 75 to 225 IU per ampoule lyophilisate.

Any gonadotropin used is preferably present in the lyophilisate preparations in a quantity sufficient to form a therapeutically useful concentration of the protein after dilution, generally for parenteral (e.g. subcutaneous or intravenous) administration, with a specified amount of an aqueous solution (e.g. distilled water for injection or sterile normal saline) to form a volume of preparation contemplated for use. As used herein, an aqueous solution is a solution containing water as a

primary, but not necessarily the only, solvent. For example, a container containing FSH may contain 1 to 1000 micrograms (μg) of FSH (e.g. 75 international units is considered a therapeutic amount). Preferably, the highest reasonable amount of protein possible will be present in a container, since the greater the amount of protein present, generally the more stable the preparation. Useful doses of gonadotropins are known to medical practitioners, and the amount included in a dose is generally dependent upon the disease state and the particular patient being treated.

Illustratively, amounts as high as 10,000 international units and as low as 15 international units of HCG have been administered. Injections ranging from 20 to 225 international units LH have been used.

In one preferred embodiment, a combination of FSH and LH or FSH and HCG are lyophilised together to from a preparation having therapeutic amounts of both of the selected gonadotropins.

B. Stabilizers

As used herein, "stabilize" is a relative term. To stabilize with a stabilizing agent or compound means the ability to prevent or delay a decrease in the protein's activity with the stabilizing agent. For example, a preparation would be deemed "stabilized" if, with the addition of a stabilizing compound ("stabilizer"), it took longer (e.g. 2 weeks instead of 1 week) to degrade at a set temperature, thus losing some of its in vivo or in vitro activity in comparison to the preparation sans the stabilizer.

A protein's activity may be determined by known methods relating to the particular protein. One measure of activity can be made by measuring the amount of (inactive) oligomers formed over time. Oligomer formation in a sample can be determined by HPSEC.

Other methods of determining the residual activity of, for example, rFSH include enzyme immunoactivity assay ("EIA") as described in U.S. Pat. Reissue No. 32,696 to Schuurs et al; a kit available under the trade designation "FSHEIA" from bioMérieux of Marcy l'Etoile 69260 Charbonnières-les-Bains, France for FSH; and in vitro bioassay of both FSH and LH as described in Mannaerts et al, "Applications of in vitro Bioassays for Gonadotropins," *Neuroendocrinology of Reproduction*, pp. 49–58 (Elsevier Science Publishers bv, Amsterdam, NL 1987).

Preferred stabilizers for use with the preparations are salts of dicarboxylic acids such as citric acid, tartaric acid, aspartic acid, and mixtures thereof. Preferred salts are the sodium, potassium, lithium and ammonium salts of such dicarboxylic acids, especially sodium and potassium salts. Another dicarboxylic acid salt is sodium glutamate. The presence of a dicarboxylic acid salt stabilizer acts to stabilize the enzymatic mixtures, especially at relatively higher temperatures over longer periods of time.

When used as stabilizers, aspartate and glutamate salts gave better recovery of activity than did citrate, isocitrate, or tartrate salts.

When a citrate salt is the selected stabilizer, a ratio of citrate to gonadotropin in the range of 200 to 400 (mg/mg) is preferred, giving the best stabilization and recovery in the presence of some sugar. An especially preferred protein for use with sodium citrate is rFSH, due to the compound's ability to be stabilized with the stabilizer.

Concentrations of dicarboxylic acid salt stabilizers sufficient to form a solution having an ionic strength of greater than 0.050 are preferred in FSH compositions containing between 0.1 and 1000 μg of FSH. Especially preferred are those solutions having an ionic strength of between 0.250 and 0.350 which will generally stabilize, for example, rFSH stored at one month at 60° C. to yield a 75% recovery of rFSH. Calculation of ionic strength is well-known to those skilled in the art, for example see Chase, et al, *Remington's Pharmaceutical Sciences*, pp. 223–224, 228, and 233 (16th ed. 1980, Mack Publ. Co. of Easton, Pa., U.S.A.).

Concentrations of 2.5 to 17.5 milligrams per milliliter (mg/ml) of sodium citrate in solution are generally sufficient to stabilize lyophilized rFSH in the amounts described herein.

Also as depicted in FIG. 2, the weight ratio of citrate to HCG should be less than 10,000 parts citrate to 1 part HCG to achieve greater stability. A preferred stabilized lyophilized gonadotropin composition preferably contains one part by weight of a gonadotropin; and 200 to 10,000 parts by weight of a dicarboxylic acid salt.

C. Non-reducing Sugars

The compositions to be freeze-dried preferably contain a non-reducing sugar such as sucrose or trehalose. The incorporation of such a sugar, e.g. sucrose, acts to increase the "collapse (or 'shrinkage') temperature" at which the lyophilization of the solution takes place. This increase in temperature simplifies the entire freeze-drying process. An especially preferred non-reducing sugar for this use is sucrose in its amorphic state.

The amount of non-reducing sugar present in the solution to be lyophilized will generally be dependent upon the amount of dicarboxylic acid salt stabilizer present. For example, the weight ratio of non-reducing sugar to dicarboxylic acid salt will generally vary between 50:1 to 10:3 with a preferred concentration being about 3.3:1 in the case of sucrose to sodium citrate. Especially preferred is a solution containing 50 mg/ml sucrose and 14.7 mg/ml sodium citrate which also yields an optimal lyophilisate in terms of physical characteristics.

In the presently most preferred embodiments, the amount of sucrose will be sufficient to raise the collapse temperature from −38° C. to about −25° C. as determined by differential scanning calorimetry. The resulting lyophilisate "cake" remains amorphous and stable for relatively longer periods of time. D. Anti-absorption agents

Anti-adsorption agents are preferably added to the lyophilized composition to prevent adsorbance of the protein to the walls of the container in which the compositions are contained, thus preventing a possible decrease in concentration. Certain anti-adsorption agents (e.g. polysorbates) also act as "cryoprotectants" protecting the protein during the lyophilization process.

Preferred anti-adsorption agents are nonionic surfactants such as Polysorbate 20, NF (Tween 20 available from Atlas Chemical Company), Polysorbate 80, NF (Tween 80 available from Atlas Chemical Company), Brij 35 (available from ICI Pharmaceuticals of Great Britain), and Pluronic F123 (available from BASF of Ludwigshafen, W. Germany). Polysorbate 20, NF is especially preferred.

Polysorbate is preferably understood as meaning a polysorbate which meets the specification of USP/NF XXII, which is published as "*The National Formulary*", p. 1763 and 1967, Official from 1 Jan. 1990 (22nd ed., U.S. Pharmacopeial Convention, Inc. 1989).

An anti-adsorption agent or anti-adsorption agents will be present in such amounts that adsorption of the protein onto container walls, or walls of vessels during processing, is decreased. Illustratively, amounts of Polysorbate 20 sufficient to form a concentration between 0.1 and 0.2 mg/ml in the ultimate solution for use are preferred. Concentrations higher than this tend to lead to oligomer formation, and thus decreased activity.

E. Pharmaceutical Compositions

The stable lyophilized preparation of the instant invention can be prepared by admixing the selected protein in aqueous solution with a sufficient amount of a dicarboxylic acid salt stabilizer to stabilize the protein, and a sufficient amount of a non-reducing sugar to increase the collapse temperature from −38° C. to greater than −25° C. Temperatures greater than −35° C. are preferred. Optionally, the selected anti-adsorption agent may also be added. The solution is then filtered, placed into containers (e.g. one ml glass ampoules) and then freeze-dried to form a stabilized lyophilisate. Freeze-drying techniques are well-known to those of skill in the art. For more information, reference may be made to several texts, including Goldblith et al, *Freeze Drying and Advanced Food Technology*, (Academic Press, Inc., London, GB 1975). Preferred residual water content in the lyophilisate cakes are between 1 and 5%. Aseptic techniques should be used throughout the procedure. Freeze-driers are available from manufacturers such as Leybold or Edwards. Using such a procedure, or modifications thereof, several different compositions may be prepared.

An especially preferred composition contains rFSH in admixture with a stabilizer which is a salt of a dicarboxylic acid, wherein the dicarboxylic acid is selected from the group consisting of citric acid, tartaric acid, aspartic acid, and mixtures of these acids.

Another preferred lyophilized preparation contains, in admixture, a dicarboxylic acid salt stabilizer, a gonadotropin capable of stabilization by the amount of stabilizer present in the preparation, and trehalose. This preparation further include sodium biphosphate in admixture with the stabilizer, protein, and non-reducing sugar. Especially preferred salts for such preparations are sodium aspartate, sodium citrate, and sodium tartrate.

Another preferred stable lyophilized preparation contains, in admixture, a stabilizer such as a salt of tartaric or aspartic acid, a gonadotropin capable of stabilization by the amount of stabilizer present in the preparation, and a non-reducing sugar. The preparation may further include disodium biphosphate in admixture with the stabilizer, protein, and non-reducing sugar. Especially preferred non-reducing sugars are trehalose and sucrose. An especially preferred stabilizer in such preparations is sodium aspartate.

Another highly preferred stabilized lyophilisate consists essentially of a protein; a sufficient amount of a dicarboxylic acid salt stabilizer to stabilize the protein in freeze dried form; a disaccharidic non-reducing sugar; an anti-adsorption agent to prevent said protein from adsorbing onto a container containing the lyophilisate; and less than five percent residual water. In such a lyophilisate the protein will be FSH; the dicarboxylic acid salt stabilizer will be selected from the group consisting of salts of citric acid, tartaric acid, and aspartic acid; the disaccharidic non-reducing sugar will either be sucrose or trehalose, and the anti-adsorption agent will be selected from the group consisting of Tween 20, Tween 80, Brij, or pluronic acid. This lyophilisate is especially preferred since, among other things, it has been discovered that the addition of further "stabilizers," such as mannitol, maltose, or either of them actually act to destabilize the lyophilisate in terms of activity.

Methods for making parenteral preparations and intravenous admixtures are disclosed in *Remington's Pharmaceutical Sciences*, pp. 1463-1497. However, caution must be exercised since although the stabilized compositions are compatible with infusion liquids, the infusion liquid used preferably should not contain reducing sugars. The preferred pH of the resulting solution for use should be between 6 and 8, especially 7.

The invention is further explained by reference to the following EXAMPLES:

EXAMPLE I

A. Stabilization of rFSH utilizing various disaccharides

Aqueous solutions containing 150 units of rFSH were prepared. The solutions were divided into three groups and each group was mixed with (1) 50 mg/ml maltose / 14.7 mg/ml sodium citrate; (2) 50 mg/ml trehalose / 14.7 mg/ml sodium citrate and (3) 50 mg/ml sucrose 14.7 mg/ml sodium citrate. All three solutions also contained 0.2 mg/ml Polysorbate 20, NF. The three groups of solutions were freeze-dried, and the resulting lyophilisate allowed to sit for four weeks at 60° C. The lyophilisates were then tested for activity (as determined by EIA) with the following results:

Compound:	(1)	(2)	(3)
Percentage activity:	40%	89%	87%

This EXAMPLE shows that non-reducing disaccharides aid stability better than reducing disaccharides.

B. Stabilization of rFSH with Sodium Citrate

Two lyophilized samples are made. The first sample contains 75 Units rFSH, 25 mg amorphic sucrose, 7.35 mg sodium citrate, and 0.1 mg Polysorbate (Tween) 20. The second sample contains 75 Units rFSH, 25 mg amorphic sucrose, and 0.2 mg Tween 20. The pH of both samples was adjusted to 7. The first sample is stored for 3 months at 50° C., reconstituted with purified water, and analyzed by HPSEC. The resulting profile showed little oligomer formation. The second sample, not containing sodium citrate, was stored for 6 months at 50° C., reconstituted with purified water, and analyzed by HPSEC. The resulting profile showed much more oligomer formation.

The profile of the first sample showed no degradation products while the profile of the second sample showed almost exclusively oligomeric products.

EXAMPLES II-V

Other dicarboxylic acid salt stabilizers

As depicted in FIG. 1, samples containing rFSH and various stabilizers were made, lyophilized, and tested for activity after 1 month storage at 60° C. FIG. 1 depicts the correlation between ionic strength (X1000) of the particular stabilizer versus the percentage recovery (as determined by EIA).

The stabilizers tested were sodium citrate, both separately (II) and with 3.0 to 9.1 mg Na_2HPO_4 per ampule (III); sodium tartrate (IV); and sodium aspartate (V).

Concentrations were determined in terms of ionic strength as shown in FIG. 1.

EXAMPLE VI

The effect of weight ratio of citrate to gonadotropin (HCG) activity was tested. The results are shown in FIG. 2.

EXAMPLE VII

A lyophilised composition for recombinant human FSH was made containing 75 IU rFSH, 14.7 mg sodium citrate, 50 mg sucrose, and 0.2 mg polysorbate 20. The preparation is reconstituted with one ml of water for injection.

EXAMPLE VIII

A lyophilised composition for recombinant human FSH was made containing 75 IU rFSH, 75 IU LH. 15 mg sodium citrate, 50 mg sucrose, and 0.2 mg polysorbate 20. The composition is stable. The preparation is reconstituted with one ml of water for injection.

What is claimed is:

1. A stabilized lyophilized FSH composition comprising:

1 part by weight FSH and

1 to 10,000 parts by weight of at least one salt of an organic acid selected from the group consisting of salts or citric acid, tartaric acid, aspartic acid, isocitric acid, glutamic acid, and mixtures thereof,

whereby a decrease in FSH activity over time is delayed.

2. The gonadotropin composition of claim 1, further comprising a non-reducing sugar selected from the group of sucrose and trehalose, wherein the non-reducing sugar is present in an amount of about three to fifty times by weight of the organic acid salt.

3. The composition of claim 1, wherein said composition contains sufficient organic acid salt to form a solution having an ionic strength of at least 0.05 mol/l when said composition is reconstituted with water.

4. The composition of claim 1 wherein said organic acid salt is selected from the group consisting of salts of tartaric acid, aspartic acid, isocitric acid, glutamic acid, and mixtures thereof.

5. The composition of claim 2 wherein said non-reducing sugar is trehalose.

6. The composition of claim 2, wherein the non-reducing sugar is present in an amount of about three times by weight of the organic acid salt.

7. A lyophilized FSH composition comprising:

one part by weight of a FSH, said FSH being of recombinant source; and

200 to 10,000 parts by weight of at least one salt of an organic acid selected from the group consisting of salts of citric acid, tartaric acid, aspartic acid, isocitric acid, glutamic acid and mixtures thereof.

* * * * *

EXHIBIT 2

EXHIBIT 2
BRIEF DESCRIPTION OF REPRESENTATIVE
SIGNIFICANT ACTIVITIES DURING THE
REGULATORY REVIEW PERIOD

DATE	ACTIVITY	COMMENTS
01/10/96	Letter	Submission of NDA 20-582
01/18/96	Letter	Letter from Ms. Enid Galliers Acknowledging Receipt of NDA 20-582
01/22/96	Teleconference	Call to Ms. Lana Pauls Re: Trademark Review of "Follistim"
01/31/96	Teleconference	Call to Ms. Lana Pauls Re: Trademark Review of Follistim
02/06/96	Letter	Letter to Dr. Ridgely Bennett, Medical Reviewer Enclosing Copy of Portion of NDA Volume As Per His Request
02/08/96	Teleconference	Call to Dr. Gus Turner Re: Request for Copy of Application Summary, Identification of Pivotal Studies and the Protocol for Each Pivotal Study
02/09/96	Letter	Letter to Dr. Gary Barnette Re: Information Related to the Pharmacokinetic and Clinical Studies in NDA 20-582
02/09/96	Letter	Letter to Dr. Gus Turner Enclosing Copies of the Information Requested During the 2/8/96 Conversation
02/09/96	Teleconference	Call to Dr. Gary Barnette Re: Questions Related to Pharmacokinetics / Bioavailability Section of the NDA
02/12/96	Teleconference	Call to Ms. Lana Pauls Re: Fileability of NDA 20-582
02/26/96	Teleconference	Call to Ms. Lana Pauls To Follow-up on Pharmacokinetic Review Comments
03/04/96	Letter	Letter to Ms. Lana Pauls Enclosing Copies of Debarment Certification, Patent Information, Clinical / Statistical Application Summary, Labeling and Integrated Efficacy and Safety Summaries
03/04/96	Letter	Letter from Dr. Solomon Sobel Re: Questions Relating to the Biopharmaceutics Section of NDA 20-582
03/04/96	Teleconference	With Ms. Lana Pauls Re: Pharmacokinetic Review Comments and Tradename Review
04/04/96	Letter	Letter from Dr. Duu-Gong Wu Re: Trade Name For Follistim
04/04/96	Teleconference	Call to Dr. Duu-Gong Wu Re: Follistim Tradename
04/10/96	Letter	Letter to Ms. Lana Pauls Requesting a Teleconference Regarding the 3/4/96 Letter Concerning Biopharmaceutics Comments

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DATE	ACTIVITY	COMMENTS
04/18/96	Teleconference	Call From Dr. Gus Turner Re: Inspection of Clinical Study Sites
05/14/96	Teleconference	Call to Dr. Gus Turner Re: Attempts to Schedule Inspection of Clinical Study Sites
05/16/96	Letter	Letter to Dr. Solomon Sobel Amending the Chemistry, Manufacturing and Controls Section of NDA 20-582
05/16/96	Letter	Letter to the FDA - North Brunswick, NJ Office Enclosing Copies of Amendment of the Chemistry, Manufacturing, and Controls Section of NDA 20-582
05/16/96	Teleconference	With Ms. Lana Pauls Re: Amendment to Chemistry, Manufacturing and Controls Section of NDA 20-582
05/20/96	Letter	Letter to Dr. Gus Turner Re: Inspection of Clinical Study Sites
05/14/96	Letter	Letter to Dr. Solomon Sobel Re: Follistim Tradename
06/03/96	Letter	Letter to Dr. Solomon Sobel Enclosing Update of Safety Information
06/04/96	Teleconference	Call from Dr. Gus Turner Re: Arrangements for Inspection of Clinical Study Sites
06/05/96	Teleconference	Call from Dr. Gus Turner Re: Arrangements for Inspection of Clinical Study Sites
06/07/96	Letter	Letter to Dr. Gus Turner Re: Inspection of Clinical Study Sites
06/10/96	Teleconference	Call from Mr. Joseph McGinnis from FDA, North Brunswick, NJ - Re: Pre-Approval Inspection
06/11/96	Teleconference	Call from Dr. Gus Turner Re: Inspection of Clinical Study Sites
06/18/96	Inspection	Pre-Approval Inspection by Ms. Regina Brown and Mr. Joseph McGinnis
06/19/96	Teleconference	Call to Dr. Duu-Gong Woo Re: Tradename for Follistim

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DATE	ACTIVITY	COMMENTS
06/20/96	Teleconference	With Ms. Lana Pauls Re: Status of NDA Review
06/21/96	Teleconference	Call to Dr. Gus Turner Re: Clinical Study Site Inspections
06/21/96	Inspection	Ms. Erin Gode and Mr. Joseph McGinnis Resumed Pre-Approval Inspection
06/24/96	Teleconference	Call from Mr. Joseph McGinnis Confirming Request for Forensic Samples
06/24/96	Teleconference	Call to Mr. Joseph McGinnis Clarifying Nature of the Information Needed for the Forensic Testing
06/25/96	Inspection	Ms. Regina Brown, Ms. Erin Gode and Mr. Joseph McGinnis Arrived to Complete the Pre-Approval Inspection
06/28/96	Letter	Letter from Matthew H. Louis Re: Successful Pre-Approval Inspection
07/03/96	Teleconference	Call to Ms. Lana Pauls Re: Successful Pre-Approval Inspection
07/12/96	Letter	Letter to Dr. Gus Turner Re: Enclosing Information Regarding Audit of Clinical Study Site
07/16/96	Letter	Letter to Mr. Ray Abrahams Responding to Mr. Louis' Letter of 6/28/96 Re: Pre-Approval Inspection
09/20/96	Letter	Letter from Ms. Joy D. Mele Re: Request for Data Sets
09/23/96	Teleconference	Call from Ms. Lana Pauls Re: The Tradename for "Follistim"
09/24/96	Letter	Letter from Dr. Lisa Rarick Confirming Approval of Follistim Tradename
09/25/96 09/26/96	Teleconference	Call from Ms. Joy Mele Re: Data in Draft Package Insert
09/27/96	Letter	Letter to Dr. Lisa Rarick Enclosing SAS Data Sets
10/04/96	Letter	Letter to Dr. Lisa Rarick Re: SAS Data Sets and Literature References

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DATE	ACTIVITY	COMMENTS
10/08/96	Letter	Letter to Dr. Lisa Rarick Re: Change in Analytical Testing Laboratories
10/25/96	Teleconference	Call to Ms. Lana Pauls Re: Status of NDA 20-582
10/31/96	Teleconference	Call from Ms. Lana Pauls Re: CMC Section of the NDA
11/08/96	Teleconference	Call from Ms. Lana Pauls Re: Status of CMC Deficiencies
11/01/96 11/06/96	Teleconference	Call from Ms. Joy Mele Re: Data and Clinical Studies
11/04/96 11/07/96	Teleconference	Call from Ms. Kate Meaker Re: Data Found in Clinical Studies
11/12/96	Teleconference	Call to Ms. Lana Pauls Re: Status of CMC Section
11/15/96	Letter	Letter to Ms. Joy Mele Re: Data From Clinical Studies
11/18/96	Letter	Letter to Ms. Joy Mele Re: Data From Clinical Studies
11/22/96	Letter	Letter to Dr. Lisa Rarick Re: Microbiology Sections of the NDA
12/02/96 12/03/96 12/04/96	Teleconference	With Dr. Lisa Rarick, Ms.Terri Rumble and Ms. Jennifer Prochnow Re: Status of CMC Deficiency Letter
12/05/96	Letter	Dr. Lisa Rarick Re: FDA's Review of the Manufacturing Quality and Controls Section of NDA 20-582
12/11/96	Teleconference	Ms. Lana Pauls Re: Timing of the Response to the 12/5/96 Letter
12/19/96	Letter	Letter from Dr. Lisa Rarick Re: The Biopharmaceutics Section of NDA 20-582
01/06/97	Teleconference	With Ms. Lana Pauls, Dr. Gary Barnette, Dr. Angelica Dorantes Re: Dose Proportionally Studies
01/09/97	Letter	Letter to Dr. Lisa Rarick Re: Manufacturing Quality Controls Section and Biopharmaceutics Section of NDA 20-582
02/05/97	Letter	Letter to Dr. Lisa Rarick Re: Chromatography of Drug Substance
02/11/97	Letter	Letter to Dr. Lisa Rarick Amending the 1/9/97 Response

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DATE	ACTIVITY	COMMENTS
02/14/97	Letter	Letter to Dr. Lisa Rarick Further Clarifying Chromatography Issue
02/20/97	Teleconference	Call from Dr. Duu-Gong Wu Re: Color Photographs Included the 1/9/97 Response
03/11/97	Teleconference	Call from Dr. Duu-Gong Wu and Ms. Lana Pauls Re: Specification for Oxidation Drug Products and Request for Package Insert on Diskette
03/11/97	Letter	Letter to Dr. Lisa Rarick Following up on the Conversation With Dr. Duu-Gong Wu
03/11/97	Letter	Letter to Ms. Lana Pauls Enclosing Disk of the Draft Package Insert
03/21/97	Teleconference	Call from Ms. Lana Pauls Re: Status of Generic Name of the Product
03/25/97	Letter	Letter from Dr. Lisa Rarick Re: Review of Draft Package Insert
03/27/97	Teleconference	Call to Ms. Lana Pauls Re: FDA Comments to the Package Insert
03/27/97	Letter	Letter to Dr. Lisa Rarick Re: Specification / Labeling
04/02/97	Teleconference	Call from Ms. Lana Pauls Re: Generic Name, Marketing Exclusivity and FDA Response 1/9/97 Response
04/07/97	Teleconference	Call from Dr. Duu-Gong Wu Re: Status of the Generic Name, "Follitropin Beta" Also Discussed Oxidation and CMC Issues
04/08/97	Letter	Letter to Dr. Lisa Rarick Responding to the FDA Letter of 3/25/97
04/08/97 04/09/97	Teleconference	Calls From Ms. Lana Pauls Re: Inspection of Manufacturing Facility
04/10/97	Letter	Letter from Ms. Lisa Rarick Re: Non-Approvable Status of NDA 20-582
04/18/97	Letter	Letter to Dr. Lisa Rarick Re: The Non-Approvable Letter

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DATE	ACTIVITY	COMMENTS
04/21/97	Teleconference	Call to Dr. Duu-Gong Wu Re: Storage and Oxidation
04/21/97	Teleconference	Call from Dr. Duu-Gong Wu Following Up on Earlier Conversation Regarding Oxidation
05/29/97	Teleconference	Call From Dr. Duu-Gong Wu Re: Recommended Storage Condition for the Product
06/03/97	Letter	Letter to Dr. Lisa Rarick Re: Label and Storage Conditions / Bioactivity
06/27/97 06/30/97 07/02/97	Teleconference	Calls to Dr. Duu-Gong Wu and Ms. Lana Pauls Re: Status of the NDA Review
07/03/97	Teleconference	Call From Dr. Duu-Gong Wu Re: Harmonization of Labeling for FSH Products
07/08/97	Teleconference	Call from Ms. Lana Pauls Re: Package Insert
07/08/97	Teleconference	Call to Ms. Lana Pauls and Dr. Duu-Gong Wu Re: Package Insert
07/10/97	Teleconference	Call to Ms. Lana Pauls Following Up on FDA's Request for Labeling With Regard to Follitropin Beta /Follitropin Alpha
07/11/97	Letter	Letter to Dr. Lisa Rarick Re: Suggested Text for Description of Follitropin Beta / Follitropin Alpha
07/18/97	Teleconference	Call to Ms. Lana Pauls Re: Status of NDA Review
07/24/97	Letter	Letter to Dr. Lisa Rarick Re: Copies of Proposed Promotional Materials
07/25/97	Teleconference	Call to Ms. Lana Pauls to Clarify the Current Status of Package Insert Review and to Clarify Previous Labeling Modifications Requested by FDA
07/29/97	Teleconference	Call From Ms. Lana Pauls Re: Status of Package Insert Review
07/30/97	Letter	Letter to Dr. Lisa Rarick Re: Storage Conditions / Vile Filling / Expiration Dating

EXHIBIT 2
BRIEF DESCRIPTION OF REPRESENTATIVE
SIGNIFICANT ACTIVITIES DURING THE
REGULATORY REVIEW PERIOD

DATE	ACTIVITY	COMMENTS
08/06/97	Teleconference	Call from Ms. Lana Pauls Re: Package Insert
08/07/97	Teleconference	Call from Ms. Lisa Stockbridge Re: Proposed Promotional Materials
08/11/97	Letter	Letter to Dr. Lisa Rarick Re: Review of Package Insert and the Product Label
08/12/97	Teleconference	Call From Ms. Lana Pauls Re: Update on the Labeling Review
08/15/97	Letter	Letter From Dr. Lisa Rarick Re: Additional Questions Regarding the Package Insert and Labeling
08/18/97 08/21/97	Teleconference	With Ms. Lana Pauls Re: Proposed Meeting With FDA to Discuss Package Insert and Labeling Issues
08/21/97	Letter	Letter to Dr. Lisa Rarick Requesting a Meeting to Discuss the Package Insert and Labeling
08/28/97	Letter	Letter to Dr. Lisa Rarick Re: Enclosing a Revised Draft Package Insert
09/03/97	Letter	Letter to Ms. Lisa Stockbridge Re: Box / Carton Labels
09/12/97	Letter	Letter from Ms. Lisa Stockbridge Re: Proposed Promotional Materials
09/15/97	Teleconference	With Ms. Lisa Stockbridge Re: Proposed Promotional Materials
09/12/97 09/15/96 09/16/97	Teleconference	With Ms. Lana Pauls Re: Package Insert
09/17/97	Letter	From Ms. Lana Pauls Re: Package Insert
09/17/97	Letter	To Ms. Lana Pauls Re: Package Insert
09/17/97	Letter	From Dr. Lisa Rarick Re: Package Insert
09/18/97	Teleconference	With Dr. Lisa Rarick Re: Package Insert
09/19/97	Letter	To Dr. Lisa Rarick Re: Package Insert
09/22/97 09/23/97	Teleconference	With Ms. Lana Pauls Re: Status of NDA 20-582
09/24/97	Letter	To Dr. Lisa Rarick Re: Package Insert
09/25/97	Letter	To Dr. Lisa Rarick Re: Additional Modifications to the Package Insert
09/29/97	Letter	To Dr. Lisa Rarick Re: Final Modification to Package Insert
09/29/97	Letter	From Dr. Lisa Rarick Approving NDA 20-582

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE: :

U.S. PATENT NO. 5,270,057 :

ISSUED: December 14, 1993 :

TO: Andreas L.J. de Meere and Marinus A. De Ruiter :

FOR: STABILIZED GONADOTROPIN CONTAINING PREPARATIONS :

FROM: SERIAL NO. 914,227 :

OF: July 3, 1992 :

TRANSMITTAL LETTER FOR GENERAL POWER OF ATTORNEY

Assistant Commissioner for Patents
Box Patent Extension
Washington, D.C. 20231

Sir:

Submitted herewith is a General Power of Attorney appointing William M. Blackstone, Registration No. 29,722, Mary E. Gormley, Registration No. 34,409 and Gregory R. Muir, Registration No. 35,293 as attorneys and agents to represent Akzo Nobel N.V. with respect to all patents and patent applications for which they are the assignee. This U.S. Patent 5,270,057 was assigned to Akzo N.V. by virtue of an assignment recorded in the United States Patent and Trademark office the 20th day of March, 1991, at Reel 5640 and Frame 0930. The name of Akzo N.V. was changed to Akzo Nobel N.V. by alteration of the Articles of Association at a

General Meeting of stockholders on January 27, 1994. A copy of the Certification of Name change is being submitted for recording with respect to U.S. Patent No. 5,270,057 concurrently herewith.

Respectfully submitted,

William M. Blackstone
Attorney for Applicant
Registration No. 29,772

Akzo Nobel NV
1300 Piccard Drive, Suite 206
Rockville, MD 20850-4396

Dated: 11/24/97

GENERAL POWER OF ATTORNEY

We, Akzo Nobel, N.V., a corporation of the Netherlands, headquartered in Arnhem, The Netherlands, with its Pharmaceutical Division headquartered in Oss, The Netherlands, hereby appoint William M. Blackstone, Registration Number 29,772, Mary E. Gormley, Registration Number 34,409, and Gregory R. Muir, Registration Number 35,293, as attorneys and agents to represent us in all matters before the United States Patent and Trademark Office with respect to all patents and patent applications for which we are Assignee and/or we as Akzo Nobel N.V. or any of its affiliates are the employers of those persons named as inventors or co-inventors.

AKZO NOBEL N.V.

November 24, 1997
Date

Name: Frans G. Hermans
Title: Director, Patents
Pharma Group

November 24, 1997
Date

Name: Bert Reerink
Title: Deputy General Counsel

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

IN RE:

U.S. PATENT NO. 5,270,057 :

ISSUED: December 14, 1993 :

TO: Andreas L.J. de Meere and Marinus A. De Ruiter :

FOR: STABILIZED GONADOTROPIN CONTAINING PREPARATIONS :

FROM: SERIAL NO. 914,227 :

OF: July 3, 1992 :

DECLARATION ACCOMPANYING APPLICATION OF AKZO NOBEL N.V. FOR EXTENSION OF THE TERM OF U.S. PATENT NO. 5,270.057 UNDER 35 U.S.C. §156

Assistant Commissioner for Patents and Trademarks
Box Patent Extension
Washington, D.C. 20231

Sir:

I, William M. Blackstone, declare as follows:

THAT I am Attorney for Applicant, Akzo Nobel N.V. (hereafter "Akzo"), and that I am authorized to obligate Akzo with respect to this matter by virtue of a General Power of Attorney dated November 24, 1997, the telefaxed original of which is submitted herewith;

THAT I have reviewed and I understand the contents of the application of Akzo, dated November 24, 1997, which is being submitted herewith for extension of the term of United States Patent No. 5,270,057 under 35 U.S.C.§156.

THAT I believe that United States Patent No. 5,270,057 is subject to an extension pursuant to 37 C.F.R.§1.710;

THAT I believe that the extension of the term of United States Patent No. 5,270,057 of 193 days that is being claimed by Akzo is justified under 35 U.S.C. §156 and applicable regulations; and

THAT I believe that the patent for which extension is being sought meets the conditions for extension of the term of a patent as set forth in 37 C.F.R.§1.720.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent for which this application is submitted herewith or any extension of patent term granted thereon.

Signed this 24th day of November, 1997 at Rockville, Maryland, U.S.A.

William M. Blackstone
Attorney for Applicant
Registration No. 29,772

Akzo Nobel NV
1300 Piccard Drive, Suite 206
Rockville, MD, 20850-4396
(301) 948-7400

TO THE HONORABLE COMMISSIONER
OF PATENTS AND TRADEMARKS
WASHINGTON DC 20231

REQUEST FOR RECORDING AND COVER SHEET UNDER 37 CFR 3.28 AND 3.31
Please record the attached original document or copy thereof.

1. Name of party conveying the interest:
   Akzo N.V.

2. Name and address of party receiving the interest:
   Akzo Nobel N.V.
   Velperweg 76
   6824 BM Arnhem
   The Netherlands

3. Nature of conveyance:
   [ ] Assignment [X] Change of Name [ ] Other

   Execution Date: February 25, 1997

4. Patent Application Number:
   Patent Number: 5,270,057

5. Address correspondence concerning this Request to: William M. Blackstone, Akzo Nobel N.V., 1300 Piccard Drive, Suite 206, Rockville, MD 20850.

6. Total number of applications or patents involved:

   1 @ $40.00 = $40.00

7. Total fee under 37 CFR 3.41: $ 40.00

8. Please charge the total fee under 37 CFR 3.41 ($40.00) to our Deposit Account No. 02-2334. In the event any fees are required with this paper, please charge our Deposit Account No. 02-2334 for which purpose duplicate copies are enclosed.

9. To the best of knowledge and belief of the undersigned, the foregoing information is true and correct and any attached copy is a true copy of the original document.

10. Date: 11/24/97

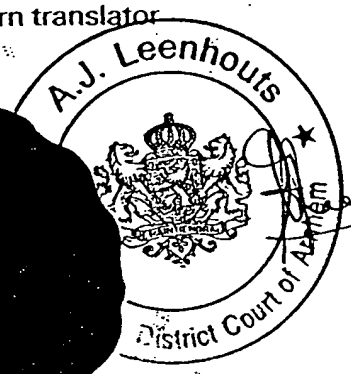
William M. Blackstone
Name: William M. Blackstone
Registration Number: 29,772

Total number of pages including cover sheet, attachments and document 5

I, Dr. Anna Jacoba Leenhouts, a sworn translator, of Doorwerth, Province of Gelderland, do hereby solemnly and sincerely declare that the following is a full, true and faithful translation, made by me, of the alteration of the company's articles.

Given under my hand and seal in witness hereof,
this 15th day of April, 1994.

Anna Jacoba Leenhouts
sworn translator



This day the twenty-fifth of February Nineteen Hundred and Ninety-Four there appeared before me, CORNELIS VENEMANS, Notary Public, residing at Arnhem:
Christiaan Bruno Burghouts, Esq., jurist, residing at 6874 AW Wolfheze, Wolfhezerweg 120/35, by his declaration born at Amersfoort on the ninth of June Nineteen Hundred and Thirty-Seven and married.

introduction

The party declared as follows:
In the general meeting of stockholders of **Akzo N.V.**, a limited liability company having its registered office at Arnhem - said company being hereinafter referred to as the company - which general meeting was held at Amsterdam on the twenty-seventh of January Nineteen Hundred and Ninety-Four, it was decided to make an alteration in the company's articles of association, the party having been authorised during said meeting to apply for a certificate of incorporation for the draft of this instrument and to lay down the alteration of the articles by a notarial instrument.
The company has its registered office situated at Velperweg 76, 6824 BM Arnhem and is registered under the Business Names Act at Arnhem under No. 7809.
The company's articles of association were most recently altered by an instrument on the twenty-fourth of August Nineteen Hundred and Ninety before me, Notary Public, a certificate of incorporation having been granted on the draft of the instrument by order of the eighth of August Nineteen Hundred and Ninety, No. N.V. 12517.
A certificate of incorporation was granted on the draft of this instrument by order of the first of February Nineteen Ninety-Four, No. N.V. 12.517, which draft instrument together with the order issued thereon is annexed to this instrument.

alteration of the articles

The party accordingly declared that he was altering article 1 of the company's articles of association to read as follows:
"Article 1.
The name of the company is **Akzo Nobel N.V.**"

in conclusion

The party is known to me, Notary Public.

OF ALL OF WHICH THE PRESENT RECORD HAS BEEN MADE,
in single copy, executed at Arnhem, on the day mentioned in the title of this instrument.
A concise statement of the contents of this instrument having been made to him, the party has stated that he has been informed of the contents of this instrument and does not wish it to be read out in full.
Immediately upon its having been read in concise form this instrument was signed by the party and me, Notary Public.

(signed:) C.B. Burghouts, C. Venemans

I CERTIFY THE ABOVE A TRUE COPY OF THE ORIGINAL SEEN BY ME:

DE CAMPENHOUT

DIRKZWAGER

ADVOCATEN NOTARISSEN

MR G VENEMANS
MR A J RIJBERS
MR M O JANSEN
MR A J SCHELLINGERHOUT
NOTARISSEN TE ARNHEM

AFSCHRIFT van de akte houdende
WIJZIGING VAN DE STATUTEN VAN:

AKZO N.V.
(na statutenwijziging genaamd:
AKZO NOBEL N.V.),
gevestigd te Arnhem
(N.V. nummer 12517)

Akte d.d. 25 februari 1994

VELPERWEG 26
6824 BJ ARNHEM
TEL. 085-538300

doss 9400232
ged. stw
ve/jh

Heden vijfentwintig februari negentienhonderd vierennegentig -- verscheen voor mij, mr CORNELIS VENEMANS, notaris ter --------- standplaats Arnhem: ----------
de heer mr Christiaan Bruno Burghouts, jurist, wonende te ----- 6874 AW Wolfheze, Wolfhezerweg 120/35, geboren te Amersfoort -- op negen juni negentienhonderd zevenendertig en ongehuwd. -----
---------- inleiding ----------
De comparant verklaarde: ----------
In de algemene vergadering van aandeelhouders van de naamloze - vennootschap Akzo N.V. met statutaire zetel te Arnhem, -------- - deze vennootschap hierna te noemen de vennootschap -, -------- welke algemene vergadering werd gehouden te Amsterdam op ------ zevenentwintig januari negentienhonderd vierennegentig, werd -- besloten tot wijziging van de statuten van de vennootschap, --- terwijl hij, comparant, daarbij werd gemachtigd op het ontwerp van deze akte de ministeriële verklaring van geen bezwaar aan - te vragen en om de statutenwijziging bij notariële akte vast -- te leggen. ----------
De vennootschap houdt kantoor aan de Velperweg 76 te 6824 BM -- Arnhem en is ingeschreven in het Handelsregister te Arnhem ---- onder nummer 7809. ----------
De statuten van de vennootschap werden laatstelijk gewijzigd -- bij akte op vierentwintig augustus negentienhonderd negentig -- voor mij, notaris, verleden, op het ontwerp van welke akte ---- bedoelde verklaring van geen bezwaar werd verkregen bij ------- beschikking van acht augustus negentienhonderd negentig, ------ nummer N.V. 12517. ----------
Op het ontwerp van deze akte is de ministeriële vrklaring van - geen bezwaar verleend bij beschikking van één februari -------- negentienhonderd vierennegentig nummer N.V. 12.517, welke ----- ontwerp-akte met de daarop gestelde beschikking aan deze akte - wordt vastgehecht. ----------
---------- statutenwijziging ----------
De comparant verklaarde dienovereenkomstig artikel 1 van de --- statuten van de vennootschap te wijzigen, zodanig dat dit komt te luiden als volgt: ----------
"Artikel 1. ----------
"De naam van de vennootschap is Akzo Nobel N.V." ----------
---------- slot akte ----------
De comparant is mij, notaris, bekend. ----------
---------- WAARVAN AKTE, in minuut opgemaakt, is verleden te Arnhem op de datum, in het hoofd dezer akte vermeld. ----------
Na zakelijke opgave van de inhoud van deze akte aan de -------- comparant heeft deze verklaard van de inhoud van deze akte te - hebben kennisgenomen en op volledige voorlezing daarvan geen -- prijs te stellen. ----------
Vervolgens is deze akte na beperkte voorlezing door de -------- comparant en mij, notaris, ondertekend. ----------

(getekend:) C.B. Burghouts, C. Venemans.

UITGEGEVEN VOOR AFSCHRIFT: